RESEARCH COMMUNICATION

Human Papillomavirus Genotype Distribution in Cervical Cancer in India: Results from a Multi-center Study

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Abstract

The prevalence of HPV genotypes in cervical cancer differs in various regions, though types 16 and 18 generally account for the majority. Knowledge of HPV genotypes in cervical cancer covering the diverse Indian population is important in consideration of the potential future impact of HPV prophylactic vaccination and HPV-based screening strategies. To determine HPV genotype distribution in cervical cancers representing different regions a total of 278 cervical cancer cases were enrolled from cancer centers in North, East, Central and South India. Cervical scrape specimens were tested for HPV DNA using the MY09/11 L1 consensus PCR method followed by sequencing for genotyping, as well as for HPV mRNA utilizing the PreTectTM HPV-Proofer assay. In instances of negative or discrepant results between the two tests, biopsy specimens were tested. HPV DNA and/or mRNA were detected in 91.7% of the cases. Genotype 16 was the most common type, detected alone in 59.4% and in association with type 18 in 3.6% of cases. Genotype 18 was detected as a monotype in 13.3% cases. In total, types 16 and 18 alone or in co-infection with each other were detected in 76.3% cases. Genotype 33 was the third most common type. Overall, genotypes 16, 18, 31, 33, and 45 were the five most common types, detected in 87.1% of the total cases. There were no significant regional differences. In conclusion, the currently available HPV prophylactic vaccines targeting types 16 and 18 have the potential to reduce the burden of cervical cancer in India by over 75%.

Key Words: Cervical cancer - India - HPV genotype - PreTect™ HPV-proofer

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Introduction

Identification of human papillomavirus (HPV) as the necessary cause of cervical cancer and subsequent development of effective vaccines against the virus has created considerable optimism world-wide, and has major implications for both primary and secondary prevention strategies. HPV types 16 and 18 have been attributed to nearly 70% of cervical cancer world-wide by the highly standardized IARC case-control studies (Munoz et al., 2004). Accordingly, the currently available vaccines have been developed targeting these two high-risk oncogenic types. The remaining fraction of cervical cancer is attributed to types 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73, 82 (Munoz et al., 2003). The prevalence and distribution of HPV types in the general population as well as in cervical neoplasias vary with geographic regions and by grade of disease (Clifford et al., 2003; Clifford et al., 2005; Smith et al., 2007., Clifford et al., 2008; Insinga et al., 2008). Although several studies have been carried out focussing on the above subject, the information concerning genotype distribution in invasive cervical cancers is important because that is the key to estimate the protection that will be offered against cervical cancer by the currently available vaccines. This also serves as a guide to determine what other types should be included in the future vaccines so as to provide maximum protection. In addition, knowledge of genotype distribution in cervical cancer is important to assess the potential future impact of HPV-based screening strategies for the prevention of cervical cancer.

India has the highest global burden of cervical cancer. Among middle-aged Indian women, cervical cancer is the most common cancer, particularly in the rural areas (Chopra 2001). It is estimated that 132,000 new cases of cervical cancer and 74,000 deaths occur each year (Bhatla et al., 2008). As with all developing countries, organized cervical cancer screening programs have not been feasible in India.

There have been many studies in India on the prevalence and distribution of HPV genotypes in the general population to cervical dysplasia and invasive

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cervical carcinoma, albeit with limitations. A recently published meta-analysis of HPV genotype distribution in women from South Asia by Bhatla et al listed six Indian studies investigating the prevalence of a broad spectrum of HPV types other than 16 and 18 in invasive cervical cancers (Bhatla et al., 2008). Five of these studies were based in South India with one of them including some patients from the eastern region; the sixth was conducted in North India (Munirajan et al., 1998; Franceschi et al., 2003; Satish et al., 2004; Sowjanya et al., 2005; Peedicayil et al., 2006; Bhatla et al., 2006). While these studies included a combined total of 558 cases of cervical carcinomas and provided valuable cumulative data on HPV genotype distribution in cancer of the cervix in India, they covered only limited regions and thus can not be considered representative of the country (Bhatla et al. 2008). Given India's vast land area and its large diverse population, there is need for further studies on HPV genotype distribution in cervical carcinomas representing various regions for accurately predicting the potential future impact of HPV vaccination (Sowjanya et al., 2005; Bhatla et al., 2006). The primary objective of the present study was therefore to determine HPV genotype distribution in representative cases of cervical cancer from different regions of the country simultaneously using a uniform protocol for sample collection and testing, and standardized histological confirmation of the lesions with testing performed in a central laboratory.

We utilized the MY09/11 L1 consensus primer PCR method for amplification of the HPV L1 region followed by nucleotide sequencing for genotyping, and the PreTect™ HPV-Proofer assay (NorChip AS, Klokkarstua, Norway) which identifies type-specific E6/E7 mRNA transcripts of five major high-risk HPV types, 16, 18, 31, 33, and 45 (Molden et al., 2007). One limitation of L1 based amplification assays is related to HPV integration into the host genome, an event which is frequently seen in cervical carcinomas, and the integrated status of HPV may lead to false negative results with L1-based HPV DNA tests. HPV integration, however, does not affect tests directed towards the E6/E7 region, which is strength of the PreTect™ HPV-proofer assay, in addition to the fact that it detects mRNA rather than DNA, reflecting oncogenic activity of the virus. While many studies have been carried out on the relative performance of the PreTect™ HPV-Proofer assay in Europe, the usefulness of this test targeting only five genotypes in an Asian setting is not known (Cuschieri et al., 2004; Sotlar et al., 2004; Molden et al., 2005; Lie et al., 2005). Therefore, application of the PreTect™ HPV-Proofer was included to ascertain the range of detection and to assess whether the detection rate of cervical cancer can be improved through mRNA testing.

Materials and Methods

Study population and collection and handling of cervical specimens

Women with histology confirmed cervical cancer were enrolled consecutively from tertiary care cancer centres in four cities representing different regions of the Indian subcontinent. These centres were Chittaranjan National Cancer Institute, Kolkata (Eastern India), Rajiv Gandhi Cancer Institute & Research Center, Delhi (North), RST Regional Cancer Center, Nagpur (Central), and Kidwai Memorial Institute of Oncology, Bangalore (South). Patients with history of previous treatment for cervical pre-cancer lesions or cancer were excluded. A written informed consent in local vernacular was obtained from each patient prior to enrolment in the study. Research Ethics Committees of the participating institutions approved the study protocol.

All cases underwent the standard procedures to confirm the diagnosis of cervical cancer and establish the stage of the disease as per standard of care in participating study centres. Staging was based on International Federation of Obstetrics & Gynaecology (FIGO) criteria. During clinical examination, the gynecologist first collected cervical scrapes using a broom-type brush and the ThinPrep PreservCyt™ collection medium containing methanol-based fixative solution (Thinprep, Cytyc Corporation, Marlborough, MA, USA), according to the manufacturer's instructions. In addition, two small pieces of tissue were taken from the tumor or the most abnormal looking area of the cervix using punch biopsy forceps. The first tissue piece was placed in PreservCyt™ medium and the second in a 5% formaldehyde solution. All specimens were held under refrigeration and couriered on ice packs to Molecular Diagnostics & Genetics division of Reliance Life Sciences, Mumbai. This is an accredited laboratory by the National Accreditation Board for Laboratory Testing and Calibration of India, and served as the central reference laboratory for the study.

Upon receipt, 2 ml of the cervical scrape specimens in PreservCyt was aliquoted for HPV DNA detection and genotyping by PCR, and stored at -80 ˚C until testing. Two 6 ml aliquots were pelleted and stored in RNase-free cryotubes at -80˚C until testing by mRNA by the PreTect HPV-Proofer assay. Cervical tissues in PreservCyt were stored at -80 ˚C and that in formaldehyde at room temperature. The PCR DNA assay was performed within a month of specimen collection and the PreTect HPV-Proofer assay was undertaken only after all specimens were accumulated. The maximum time interval between specimen collection and testing was 9 months.

HPV DNA detection and genotyping

For HPV DNA detection by PCR, 2ml of the cervical scrape specimen in PreservCyt was used. DNA amplification of the HPV L1 region was performed with the consensus MY09/11 PCR primers. The process involved 30 cycles of denaturation, amplification and extension to yield a final product of 451 bp which was visualized by agarose gel electrophoresis. Molecular weight markers were used to size the specific fragment. Positive and negative controls were included and the specimen adequacy was assessed by verifying the presence of human genomic DNA by PCR amplification of the human β-globin gene. All HPV DNA negative specimens were retested for verification of the initial result. For HPV genotyping, the PCR products were purified using the Millipore Montage SEQ96 Sequencing Reaction Cleanup

Kit (Millipore Corp. Billerica, MA, USA). Nucleotide sequencing was performed by Sanger's dideoxy nucleotide sequencing method, using the Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) (Sanger et al., 1977), with 5 pmol of MY09/11 as the sequencing primers. The sequence obtained was analyzed in silico on ABI 3100 Genetic Analyzer (Applied Biosystems). For the determination of HPV type, the results were compared with documented sequences available in the Genebank database using the National Center for Biotechnology Information BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Type assignment was done when the base identity was greater than 90% over a fragment of at least 60 nucleotides.

HPV E6/E7 mRNA detection

For HPV E6/E7 mRNA detection by the PreTect HPV-Proofer assay, one aliquot of the pelleted cervical scrape specimen was utilized, and RNA extracted with RNeasy columns (QIAGEN Ltd. Crawley, UK). PreTect HPV-Proofer is based on multiple nucleic acid sequence based amplification (NASBA) technology and utilizes molecular beacon probes for real-time detection of types 16, 18, 31, 33, and 45 (Molden et al., 2007).

A PreTect analyser fluorescence reader (NorChip AS) was used for detection of the accumulated mRNA product with the PreTect Analysis Software for analyzing the fluorescence profiles. To verify the integrity of RNA in the specimens, a primer set and probe directed against the human U1 small nuclear ribonucleoprotein (sn-RNP)-specific A protein (U1A) mRNA were used. Standardized artificial oligos corresponding to the respective viral sequences are provided in the test kit and were included as positive controls for each of the five HPV types. Water was used in all cases as a negative control.

Testing of cervical tissues

In all instances when the cervical scrape specimens tested negative by both the PCR and the PreTect HPV-Proofer assay, or yielded discordant results between the two tests, the cervical tissue specimens stored in PreservCyt solution were tested by both PCR and PreTect HPV-Proofer. For this, homogenized lysates from the tissue were processed and analyzed following the same procedures as described above.

Cervical Histology

The original cervical biopsy results were reviewed at the participating centers, and the final histology reports from participating centres were accepted as the gold standard to compare the results of the PCR and the PreTect HPV-Proofer assay.

Statistical methods

The descriptive statistics for the continuous variables are given as mean with standard deviations while that for categorical data are given as frequency distribution with confidence interval where applicable. Fisher's Exact Test was used to evaluate the significance of difference between proportions. Agreement between the test results was assessed by Kappa coefficient.

Table 1. Demographic Parameters of Patients and Stage Distribution of Cervical Cancer (N=278)

Regional distribution of cases		
South India		69 (24.8%)
North India		46 (16.5%)
East India		88 (31.7%)
Central India		75 (27.0%)
Mean age (years)		51.4 [S.D. 11.2; C.I. 10.3-12.2]
Mean age at marriage (years)		17.9 [S.D. 3.8; C.I. 3.5-4.2]
Number with >5 pregnancies		64 (23.0%)
Post-menopausal		200 (72.1%)
Stage Distribution*	Stage I	32 (11.5%)
	Stage II	82 (29.5%)
	Stage III	150 (54.0%)
	Stage IV	6 (2.2%)
	Unknown	8 (2.8%)

* International Federation of Obstetrics & Gynaecology (FIGO) staging

Results

A total of 278 women with cervical cancer were enrolled between 1 April 2007 and 31 January 2008. The regional distribution of the patients enrolled was fairly equal in three of the four regions with the northern region enrolling a relatively smaller number of patients. The study population included 262 (94.2%) squamous cell carcinomas (SCC), 11 (4%) adenocarcinomas (ADC), four undifferentiated cancers, and one case of mixed Mullerian tumour. The distribution of SCC in North India was relatively lower (84.8% versus 94.2% in East, 94.2% in South and 100.0% in West) and that of ADC relatively higher (10.9% versus 3.5% in East, 4.3% in South and 0% in West) compared to the other three regions. This difference was statistically significant (p=0.02). The mean age of the patients was 51.4 years (SD 11.2). At the time of diagnosis, 156 (56.2%) of the patients had FIGO stage III or IV disease. The regional distribution, demographic profiles and stage of disease of the 278 patients are summarized in Table 1.

The cervical scrape suspensions from all 278 cases were tested for HPV DNA by PCR followed by sequencing. Of these, 232 (83.4%) tested positive for HPV DNA. In this series, specimens from all 278 cases were positive for the β-globin DNA control signifying adequacy of the test samples. The cervical scrape specimens from 276 cases were tested for E6/E7 mRNA by the PreTect HPV-Proofer assay; specimens from two cases could not be tested as these were aged upon receipt at the laboratory and considered unsuitable for testing. Of the 276 cases, 229 (83.3%) tested positive for E6/E7 mRNA. mRNA of the human U1A gene was positive in all 276 specimens indicating that there were no false negative results due to degradation of RNA in the samples. PCR was repeated selectively on cervical tissue samples from 55 cases because either the cervical scrape specimens tested negative by PCR or by the PreTect HPV-Proofer assay or both, or there was discordance between the two test results. In this series, samples from four cases were β-globin negative and considered inadequate for PCR analysis. Of the remaining 51 cases, PCR detected HPV DNA in 15 cases. The PreTect HPV-Proofer assay was repeated on

Table 2. HPV Genotypes Detected In Cervical Cancers

HPV GENOTYPE	Number of Cases* (%)
16	165 (59.4)
18	37 (13.3)
31	3 (1.1)
33	11 (4.0)
45	5 (1.8)
52	3 (1.1)
53	1 (0.4)
56	4 (1.4)
59	1 (0.4)
62	1 (0.4)
67	1 (0.4)
69	1 (0.4)
73	1 (0.4)
16+18	10 (3.6)
16+18+45	1 (0.4)
16+31	4 (1.4)
16+52	2 (0.7)
33+58	3 (1.1)
33+62	1 (0.4)
Negative	23 (8.2)
Total	278

*Based on testing of cervical scrape specimens and biopsies for HPV DNA and mRNA by PCR and the PreTect HPV-Proofer assay, respectively. Biopsy specimens were tested only in instances of negative or discrepant results with the two tests.

48 cervical tissue samples for the same reason as above and all samples were found to be satisfactory for mRNA analysis. Of these, PreTect HPV-Proofer detected mRNA in 8 cases. Overall, multiple genotypes were detected in 11/276 cases by PreTect HPV-Proofer whereas PCR followed by sequencing did not detect multiple infections in any. The two tests detected different HPV types in nine instances in specimens from the same patient, and these results were confirmed by repeat tests. For final analysis, these patients were considered to have multiple infections with combination of all types detected. When taking into consideration all results derived from both cervical scrape and tissue specimens, HPV was detected in 255/278 (91.7%) cases by at least one of the tests, with 21 (7.6%) cases having multiple infections (Table 2).

In terms of overall genotype distribution, type 16 was detected alone in 165/278 (59.4%) cases and in association with type 18 in 10 (3.6%) cases. Type 18 was detected as a monotype in 37 (13.3%) cases. Thus, types 16 and 18 were detected alone or in association with each other in 212 (76.3%) of the 278 cases (Table 2). Type 33 was detected alone in 11/278 (4.0%) cases and in association with other types in four (1.5%) cases. Types 31 and 45 were detected alone or in association with other types in seven (2.5%) and six (2.2%) cases, respectively. Overall, genotypes 16, 18, 31, 33, and 45 were the five most common types, detected in 87.1% (242/278) of the cases, and in 94.9% (242/255) of the HPV positive cases. The distribution of the genotypes is shown in order of frequency in Figure 1. Of the 262 cases of SCC, HPV was detected in 243 (92.7%) and genotypes 16, 18, 31, 33 or 45, alone or in combination with each other, were the most common types accounting for 85.5% (224/262). HPV was detected in 9 of the 11 (81.8%) ADC cases and the same five genotypes accounted for all positive cases. The

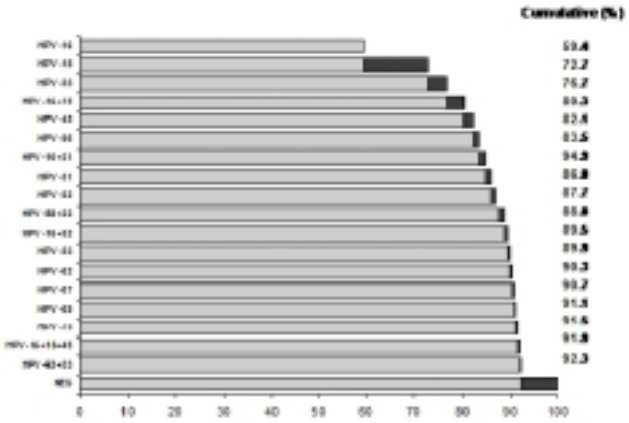

Figure 1. HPV Genotype Distribution in Cervical Cancer in Order of Frequency

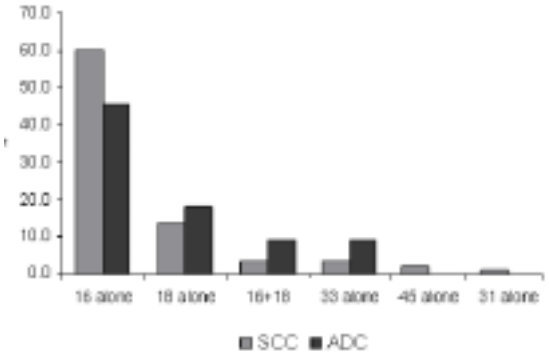

Figure 2. The Relative Proportions of the Five Most Common Genotypes in Squamous Cell Carcinomas (SCC) and Adenocarcinomas (ADC)

distribution of the five genotypes in SCC and ADC is shown in Figure 2. Type 18 was detected, alone or in association with other types, in a higher proportion (27.3%) of ADC compared with SCC (17.2%). However, the difference was not statistically significant (P = 0.41). This could be attributed to the low number of ADC cases in the study population. Regardless, genotype 16 was the most common type, detected alone or in combination with other types, in both SCC and ADC, accounting for 66.4% (174/262) and 54.5 % (6/11), respectively. Of the four undifferentiated cancers, one was negative for HPV. Of the remaining, two were positive for type 16 and one was positive for type 33. The single case of mixed Mullerian tumor was negative for HPV by all tests. The majority of women with cervical cancer were in the age group 40 to 59 years. The distribution of the five common genotypes

Table 3. Age Group-Specific Distribution of the Five Most Common HPV Genotypes in Cervical Cancer

Age group	HPV genotype 16 (+31,45,52)	18	16/18	33 (+58,62)	45	31
20-29	3 (1.7)	1 (2.7)	0	0	0	0
30-39	17 (9.9)	5 (13.5)	0	2 (13)	0	1 (33)
40-49	53 (30.8)	16 (43.2)	5 (50)	4 (27)	0	1 (33)
50-59	57 (33.1)	6 (16.2)	4 (40)	3 (20)	3 (60)	1 (33)
60-69	29 (16.9)	6 (16.2)	1 (10)	4 (27)	0	0
70-79	12 (7.0)	1 (2.7)	0	2 (13)	2 (40)	0
80+	1 (0.6)	2 (5.4)	0	0	0	0
Total	172	37	10	15	5	3

Table 4. Cervical Cancer Stage-Specific Distribution of the Five Most Common HPV Genotypes*

Stage	HPV genotype						%
	16 (+31,45,52)	18	16/18	33 (+58,62)	45	31	+ve
I	20 (62.5)	6 (18.7)	1 (3.1)	2 (6)	0	0	90.6
II	47 (57.3)	12 (14.6)	3 (3.7)	5 (6)	1 (1)	2 (4)	85.4)
III	97 (64.7)	17 (11.3)	6 (4.0)	8 (5)	4 (3)	0	88.0
IV	4 (66.7)	1 (16.7)	0	0	0	1 (17)	100

*Stage unknown = 8 cases

stratified by age group and stage of disease is shown in Tables 3 and 4, respectively.

The HPV type distribution in cervical cancer in the four regions studied is shown in Table 5. HPV 16 and 18, either alone or in association with each other, accounted for 73.9% of the cases in South India, 78.3% in North India, 76.1% in East India and 77.3% in Central India. ($X2 = 0.35$; $P = 0.99$). The prevalence of HPV 33, alone or in combination with another type, was higher in South and Central India at 10.1% and 6.7%, respectively, compared with North and East India at 2.2% and 2.3%, respectively. The proportion of HPV-negative cervical cancers was highest at 15.2% in North India and lowest in Central India at 4.0% ($p = 0.04$).

There was a good agreement ($\kappa = 0.79$) between PCR and the PreTect HPV-Proofer assay to identify the HPV types 16, 18, 31, 33, 45, the five genotypes detectable by PreTect HPV-Proofer. For this analysis, the results of PreTect HPV-Proofer showing multiple types were considered to be in agreement with the PCR results if PCR could detect any one of the types involved.

Discussion

India with its large population has considerable diversity with overlapping ethnicity and cultural differences. Our study population represented four major regions in India, and we studied a fairly large number of cervical cancer cases which amounted to about 50% of the combined total of 558 cases derived from 6 previous Indian studies which were included in the meta-analysis by Bhatla et al (Bhatla et al., 2008).

Table 5. Regional Variation in HPV Genotype Distribution in Cervical Cancer

HPV type	Number of cases by study regions			
	South (%)	North (%)	East (%)	Central (%)
16	37 (53.6)	30 (65.2)	56 (63.6)	42 (56.0)
18	13 (10.9)	5 (10.9)	6 (6.8)	13 (17.3)
31	0	0	3 (3.4)	0
33	5 (7.2)	1 (2.2)	2 (2.3)	3 (4.0)
45	1 (1.4)	0	3 (3.4)	1 (1.3)
52	2 (2.9)	0	0	1 (1.3)
53	1 (1.4)	0	0	0
56	1 (1.4)	0	0	3 (4.0)
59	0	0	0	1 (1.3)
62	0	1 (2.2)	0	0
67	0	1 (2.2)	0	0
69	0	0	1 (1.1)	0
73	0	0	0	1 (1.3)
16+18	1 (1.4)	1 (2.2)	5 (5.7)	3 (4.0)
16+18+45	1 (1.4)	0	0	0
16+31	0	0	2 (2.3)	2 (2.7)
16+52	1 (1.4)	0	1 (1.1)	0
33+58	1 (1.4)	0	0	2 (2.7)
33+62	1 (1.4)	0	0	0
Negative	4 (5.8)	7 (15.2)	9 (10.2)	3 (4.0)
Total	69	46	88	75

Our overall HPV detection rate of 91.7% in cervical cancer is consistent with previously published studies (Clifford et al., 2003). Through the simultaneous study of four different regions in India, type 16 was found to be by far the most common, with an overall prevalence of 65.5% when taken into account both single and multiple infections (Table 2); type 18 was the second most common type at 17.3%. Excluding coinfections with other types, types 16 and 18 alone or in association with each other were detected in 76.3%, and this is consistent with the 16/18 prevalence rate of 78.9% reported in the meta-analysis of Indian studies (Neerja et al., 2008), and 77% reported by Saranath et al in a separate Indian study of 337 cases of invasive cervical cancer (Saranath et al., 2002). Our observed proportion is higher than the estimated 16/18 attributable fraction of about 70% worldwide, though some regional variation exists (Clifford et al., 2003; Clifford et al., 2005; Clifford et al., 2006; Smith et al., 2007). It is worth noting that an updated meta-analysis indicated a lower fraction of 16/18 in the range of 65-70% in less developed countries compared to a higher prevalence of 74-77% in more developed countries (Smith et al., 2007). A recent study from France has reported types 16/18 in 82% of cervical cancers, again indicating a higher attributable fraction than what is generally estimated (Pretet et al., 2008).

The overall prevalence of HPV in SCC at 92.7% and in ADC at 81.8% was somewhat higher than the reported prevalence rates of 87.3% and 76.4%, respectively, in a meta-analysis of global studies (Clifford et al., 2003). In 262 cases of SCC in our study, types 16, 18 and 33 accounted for 66.4%, 17.2%, and 5.0%, respectively (inclusive of co-infections). This correlates with 64.8%, 14.7% and 6.4%, respectively, detected in 423 cases of SCC reported in the meta-analysis of Indian studies (Bhatla et al., 2008). Concerning other HPV types, however, some differences were seen; the prevalence of type 45 was lower in our study, 1.9% versus 6.4%, and we failed to detect any type 35 which was detected in 5% of SCC in the meta-analysis. In the 11 cases of ADC in our study, types 16, 18, and 33 accounted for 54.5%, 27.3%, and 9%, respectively. This was slightly different from the data on 29 cases of ADC reported in the meta-analysis, in which type 16 accounted for 51.7% followed by type 18 (34.5%), 31 (6.9%) and 33 (3.5%). Regardless, as reported in the meta-analysis, type 18 was found to be more common in ADC than in SCC, and although our data was based on a small number of cases, our observation in an Indian population corroborated the observed global trend of a relatively higher prevalence of HPV 18 in ADC than in SCC (Clifford et al., 2003; Smith et al., 2007). Since types 16 and 18 are the targets for the currently available cervical cancer vaccines, we also looked into the regional variations in proportions of cancers

attributable to these two types. The distribution was almost similar across the regions except in South India where the figure was comparatively lower (Table V). The meta-analysis of Indian studies showed a similar pattern of lower relative proportion of 16 and 18 in South India compared with North India (77.2% versus 84.8%) (Bhatla et al., 2008). All previous studies from South India that investigated a broad spectrum of HPV genotypes identified type 33 as the third most common type associated with cervical cancer, its prevalence ranging from 5.6% to 9.5% (Franceschi et al., 2003; Sowjanya et al., 2005; Peedicayil et al., 2006). We made a similar observation, and our rate of 5.5% was consistent with the above data. However, type 33 was detected more frequently in South and Central India compared to East and North India at 8.3% versus 2.2%. This appears to correlate with a lower prevalence of type 33 at 1.9% reported in a study in North India (Bhatla et al., 2006). Genotypes 52 and 58 have generally been reported to be more common in Asia than in other regions of the world (Clifford et al., 2003; Bao et al., 2008; Hong et al., 2008). However, according to the meta-analysis of Indian studies, the prevalence of HPV 52 and 58 appears to be lower compared to other Asian countries, which is supported by our study, where HPV 52 and 58 were detected in only three cases each. Although types 53 and 66 are classified as "probable high-risk" genotypes (Munoz et al., 2003), we detected type 53 in a single case only and failed to detect 66 in any.

Given the high prevalence of a few genotypes and peak disease prevalence in the age group 40-59 years, we did not find any significant trend in age-specific distribution (Table III). HPV positivity remained uniform across the clinical stages of disease and there was no significant difference in the genotype distribution between the stages (Table IV). A number of variants of E6 and E7 genes of genotype 16 have been described, and these differ geographically and may be related to differences in disease progression (Giannoudis and Herrington, 2001). Certain variants of E6 have been detected in cervical malignancies in India with indication of their preponderance in younger women and possible association with aggressive tumor (Pillai et al., 2002). We did not however investigate the nature and distribution of type 16 E6 variants in the present study. Nevertheless, further studies are under consideration to investigate type 16 E6 variants to correlate their possible association with age of patients and stage of cervical cancer to shed more light on the underlying molecular mechanisms of oncogenesis, which in turn may have implications for HPV vaccination and HPV-based screening strategies.

HPV was accepted as the 'necessary' cause of cervical cancer after the presence of the virus in virtually all cervical cancers was convincingly proved (Walboomers et al., 1999). Yet, a meta-analysis of the worldwide prevalence of HPV in invasive cervical cancer by Clifford et al., noted that 11-17% of cervical cancers have been reported to be HPV negative. Failure to detect HPV in cervical carcinomas can be due to the absence of HPV DNA in the carcinoma cells, false negative test results due to low clinical sensitivity of the test, or absence of cancer cells in the sample. The most widely used method for HPV detection is PCR using consensus primers directed towards a highly conserved region of the L1 gene. Using this method may however in some cases of advanced neoplasias lead to false negative results due to loss of the L1 region upon HPV integration into the host genome. This may also be a likely explanation for the negative PCR results in our study. Nonetheless, considerable variations in HPV detection rates and types by different testing methods are well recognized, which is reflected by a wide range of HPV positive rates and the extent of coinfections detected in cervical cancer (Walboomers et al., 1999; Klug et al., 2008). We utilized the PreTect HPV-Proofer assay in the testing of all cervical scrape specimens and in the testing of biopsy specimens for discrepant test results. Importantly, E6/E7 mRNA testing is not influenced by the event of HPV chromosomal integration and positive results by this assay being negative by the PCR assay, may therefore reflect integration involving disruption of the L1 gene. In fact, PreTect HPV-Proofer did detect HPV mRNA in many of the PCR negative samples and with an overall detection rate of 85.9%. (The details of this will be presented in a separate paper). This was similar to the detection rate of 89% reported in a Norwegian study based on 204 cervical cancers (Kraus et al., 2006). Interestingly, although the PreTect HPV-Proofer assay cannot detect HPV types beyond 16, 18, 31, 33 and 45, we observed that this test had the same overall sensitivity as the consensus PCR method in detecting HPV in cervical cancer. This is attributable to the fact that the five genotypes 16, 18, 31, 33 and 45 targeted by the test turned out to be the five most common types in invasive cervical cancer in our study, accounting for 87.1% of the total cases and 94.9% of the HPV positive cases.

Utilizing both tests, on final analysis, the proportion of HPV negative cervical cancers came down to 8.3%. Lower HPV negative results from India were reported only from the studies in which DNA amplification was done with PGMY 09/11 primers and the line blot assay was used for genotyping, (Peedicayil et al., 2006; Bhatla et al., 2006) or a study that performed general primer-mediated GP5+/GP6+ PCR followed by hybridization of PCR products with an enzyme immunoassay using two HPV oligoprobe cocktails that, together, could detect 44 HPV types (Franceschi et al., 2003). The PCR method we utilized failed to detect multiple infections, which obviously was a technical limitation of the sequencing procedure that prevented detection from more than one target even if the PCR amplified more than one target. By the PreTect HPV-Proofer assay we identified multiple infections in 11/276 (4%) cases, but as the assay is limited to five genotypes, this is also most likely an underestimate. Regardless, our rate was about the same as that of 3.7% reported in the meta-analysis by Clifford et al, but significantly lower than the 22% reported in the French study (Pretet et al., 2008).

Our multicentre study has described the prevalence and attribution of HPV types in cervical cancer and has contributed to the existing body of knowledge on genotype distribution in cervical cancer in India; the accumulating data form a basis to consider the potential future impact

of primary cervical cancer prevention through HPV vaccination and/or secondary prevention through HPV-based screening strategies. The importance of our study and that of others on genotype distribution in cervical cancer in India is underscored by the fact there are differences, albeit minor, in HPV type distribution in different regions within India. Generally, HPV types 16 and 18 are the two dominant types with the highest attribution to cervical cancer in India, as is the case worldwide. The currently available prophylactic HPV vaccines targeting types 16 and 18 have been reported to be highly efficacious in preventing persistent infection with vaccine types and the associated cervical disease (FUTURE II Study Group, 2007; Paavonen et al., 2007). A recent report has also indicated a combined cross-protective efficacy of Merck's quadrivalent vaccine in the range of 38% for ten HPV types not included in the vaccine (Villa L, 2007). Based on the above efficacy data and our observation, and assuming 100% coverage, the currently available HPV vaccines targeting 16/18 have the potential to reduce cervical cancer burden in India by over 75%. In this regard, our data support the conclusions reached in previous Indian studies. Our projected fraction is higher than the estimated coverage in the 59-64% range indicated for Asia as a whole by Clifford's meta-analysis, and 67% (range 51.3%-70%) by Bao's meta-analysis of Asian studies. Based on our study, genotypes 31, 33 and 45 may be considered priority types for possible inclusion in second generation vaccines, and vaccines with such formulation could reduce cervical cancer burden in India by over 85%. However, the incremental benefit of adding additional genotypes should be weighed in terms of the efforts and cost involved in developing tailor-made vaccines for different regions of the world, and also the time lost before the next generation of the vaccines becomes widely available. Finally, in relation to possible cervical cancer screening strategies in India in the future, our results suggest that testing for a limited number of HPV types may be sufficient for reaching a relatively good clinical sensitivity.

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