RESEARCH ARTICLE

Nested Multiplex PCR Based Detection of Human Papillomavirus in Cervical Carcinoma Patients of North- East India

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Abstract

Background: Persistent infection of one or more of about 15 high-risk human papillomaviruses (HR-HPVs), most commonly HPV types 16/18, has a significant role in cervical cancer initiation and progression. There are limited data available from north-east India about HPV prevalence though this region has high incidence rates of cervical cancer. The aim of this study was to investigate the HPV genotypes prevalent in cervical cancer patients of north-east India. Materials and Methods: We analyzed 107 cervical cancer patient samples. Nested multiplex PCR assays were employed for detection of 13 high risk and 5 low risk HPV types. Results: HPV was confirmed in 105 samples. The presence of 6 'carcinogenic' HPV types, HPV-16 (88%), -18 (15%), -31(4%) ,-45 (3%), -59 (4%), -58(1%), and one non carcinogenic, HPV-6/11 (6%), was recorded. Among various demographic and clinical factors only tumour stage showed a statistically significant association with HPV type infection (P=0.019). Conclusions: We suggest that the most prevalent genotype is HPV-16 followed by HPV-18 in cervical carcinoma patients of the north-eastern region of India. Advanced tumour stage may be associated with increased possibility of harbouring multiple HPV genotypes.

Keywords: HPV - nested multiplex PCR - cervical cancer - North East India

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Introduction

Cervical cancer is the second most common cancer in women worldwide with 529,000 new cases and 275,000 deaths. India accounts for more than a quarter of the global cervical cancer burden with 130,000 new cases and has also one of the highest mortality rates of 75,000 deaths. In India, the age standardized cervical cancer incidence and mortality rates were 27 and 15 per 100,000 women (Ferlay et al., 2010).

The risk factors known to increase the incidence of cervical cancer are early marriage and sexual practice, delivery of the first baby before the age of 20, too many or too frequent childbirths, multiple sexual partners, poor practice of personal hygiene, low socio economic status, human papillomavirus (HPV), Herpes Simplex Virus type II, HIV positivity, use of oral contraceptive pill, tobacco smoking etc. (Raychaudhuri and Mandal, 2012). Most human papillomavirus infections regress rapidly without causing clinically significant disease but several epidemiological studies have established that the persistent infection with one of the 15 oncogenic types of HPV as the essential cause of virtually all cervical cancers and its precursors, cervical intra epithelial neoplasia (CIN) (Thulaseedharan et al., 2012). The International Agency for Research on Cancer (IARC) has classified 13 HPV types as group 1 Carcinogens (i.e. oncogenic or high risk): HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 'possibly carcinogenic' (7 genotypes): HPV26, 53, 66, 67, 70, 73, 82 and 'non carcinogenic/unknown carcinogenicity' (17 genotypes): HPV6, 11, 40, 42, 54, 55, 61, 62, 64, 69, 71, 72, 81, 83, 84, CP6108, IS39 (Bouvard et al., 2009). HPV types 16 and 18 are considered as most prevalent "high risk" types while 6 and 11 ("low-risk") types are significantly associated with benign lesions and genital warts.

Depending on the geographical locations the most consistent variation has been observed in the prevalence of HPV 16. HPV infection is a common sexually transmitted infection and both sexes can be infected with the virus

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(Broomall et al., 2010).

There are suggestions from various stakeholders for implementation of a mass HPV vaccination drive for reducing cervical cancer incidence in India. However the success of any such initiative would depend upon the availability of updated and comprehensive data on type specific prevalence of HPV from all regions of the country. Not only the geographical locations but also the cultural variations influence the sexual behavior of women and their male partners leading to differential acquisition of new HPVs (Burchell et al., 2006). Data on human Papillomavirus (HPV) type distribution in women with invasive cervical cancer (ICC) and its precursor lesions are essential to predict the potential worldwide impact of new prophylactic vaccines against HPV16/18, as well as to determine priorities for the inclusion of HPV types in future HPV vaccines and HPV-based screening tests (Smith et al., 2007). A meta analysis on HPV genotype distribution in women from South Asia found that in North India, most prevalent genotypes were HPV-16 and -18 ,-45, -59 and -33, while in the South India it was HPV-16,-18, -33, -35 and -45. Four of the first five HPV types found in India are similar to the global scenario (India—HPV-16, -18, -45, -33 and -35; Africa—HPV- 16, -18, -45, -33 and -31; Europe—HPV-16, -18, -33, -31 and-45; North America/Australia—HPV-16, -18, -31, -33 and -45; and South/Central America—HPV-16, -18, -31, -45 and -33) (Bhatla et al., 2008). Epidemiological data from north east India on the prevalence of HPV genotypes are scarce and critically insufficient. The north eastern states represent a population which is ethnically and culturally distinct from the rest of the country and might so harbour HPV genotypes which may differ. According to the National Cancer Registry Programme (India), NE-PBCR 2006-2008 report published in 2012, Aizawl district of North East region had highest age standardized rate for cervix uteri cancers in the country. Among males, five of ten north east India registry areas show a higher AAR compared to Delhi, which has the highest AAR (124.3) among older PBCRs. Aizwal district has approximately twice the AAR (249.5) of Delhi. Mizoram state as a whole also shows higher AAR (176.5) followed by Kamrup urban district (161.6). Among females, two of ten registries showed higher AAR (210 and 152.8) compared to Bangalore which has the highest AAR (139.1) among the older and newer PBCRs. In North Eastern states the PBCRs started since 2003 and this region is emerging as high incidence area for cancers of various organ sites.

The diversity of virus types and the incidence of multiple infections have made it necessary to develop reliable methods to identify the different genotypes, for epidemiological studies as well as for patient follow up. Currently, the workhorse of HPV testing in the United States is a Food and Drug Administration (FDA)–approved hybridization method, Hybrid Capture 2 (HC2; Qiagen Corporation, Gaithersburg). To date, HPV DNA detection by polymerase chain reaction–based methods have been used mainly for research. The best-validated HPV tests have reasonably comparable performance (i.e., they are more reproducible than inter pathologist's comparisons of the distinction between normal and abnormal cytology), but there are exceptions (Schiffman et al., 2011). Sotlar et al. (2004) described PCR assay had utilized the viral E6/E7 oncogenes as the primer target region. In this assay, consensus primers for first-round amplification of a broad spectrum of mucosal HPV genotypes, including all high-risk HPV genotypes, were combined with type-specific primers for nested PCR amplifications. In order to reduce the numbers of nested PCRs these primers were used in multiplex primer master mix. This PCR assay was used in the present study.

This study was designed with objective to investigate the prevalence and distribution of 13 carcinogenic and 5 non carcinogenic HPV types in cervical cancer patients from Assam and other north east states of India.

Materials and Methods

Patients

All the patients (n=107) in this study had attended as new cases in the Gynaecologic Oncology Department of Dr B. Borooah Cancer Institute, Guwahati, India between January 2011- June 2012. This study was approved by an institutional medical ethics committee of Dr B. Borooah Cancer Institute. Patient's gynecologic examination and their disease were staged as per the International Federation of Gynecology and Obstetrics (FIGO) guidelines. All enrolled patients were interviewed with informed consent about demographic, lifestyle etc data.

Collection of specimens and genomic DNA preparation

A standardized per speculum examination was performed by gynecologic oncologists. All patients were subjected to a complete clinical evaluation and directed biopsy was taken where an abnormal lesion was present. Biopsy specimens were taken for histopathological examination. All slides were reviewed simultaneously by at least 2 pathologists using a multi-headed microscope (BX51, Olympus) to confirm the histologic diagnosis. A small piece of the tissue biopsy (2-5 mm) was also collected in RNAlater™ solution (Qiagen, Germany) and stored at 4°C until further processing. Twenty five milligram of tissue was minced in sterile PBS solution (Amresco, USA), homogenized by hand held plastic micro-pestle and total genomic DNA was extracted using the QIAamp DNA mini kit (QIAGEN, Germany) as per

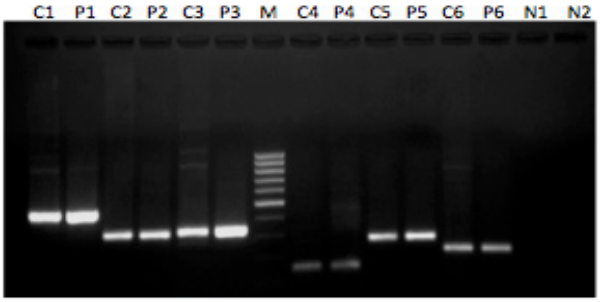

Figure 1. Gel image Showing Amplification of HPV Types in Samples and Recombinant Plasmids (positive controls). Lanes C1-C6: cervical cancer samples; P1-P6: positive controls (HPV 16, 18, 11, 45, 58 & 59 respectively); N1 & N2: Non-template controls

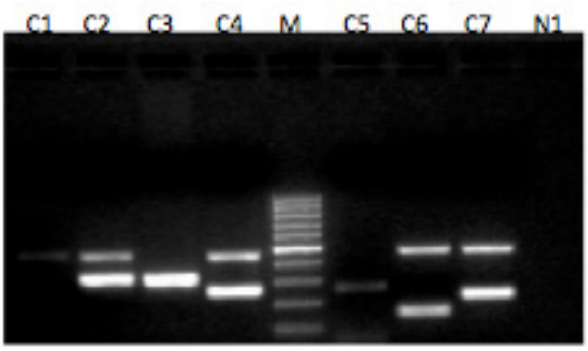

Figure 2. Agarose Gel (2%) Image of HPV Type-specific Nested Multiplex PCR (NMPCR) Product. Lane C1: HPV 16; C2: HPV 16 & 18; C3: HPV18; C4: HPV 16 & 31; M: 100bp DNA ladder; C5: HPV 31; C6: HPV 16 & 45; C7: HPV 16 & 59 and lane N1: Non-template control. Amplified PCR product size for HPV strains were - 457bp (HPV 16), 322bp (HPV18), 263bp (HPV 31), 151bp (45) and HPV59 -215bp respectively

the manufacturer's instructions. The quantity and quality of the extracted DNA was determined by a Biophotometer Plus (Eppendorf, Germany) spectrophotometer at 260/280 nm and 260/230 nm.

HPV plasmid DNA

Recombinant plasmids containing HPV DNA were kindly provided by E.-M. de Villiers, Deutsches Krebsforschungszentrum, Heidelberg, Germany (genotypes 11, 16, 18, 45, and 51); M. Favre, Institut Pasteur, Paris, France (genotypes 33 39, 42, 66, and 68); and T. Matsukura, National Institute of Health, Tokyo, Japan (genotypes 58 and 59). These were used as positive controls in the PCR assay.

Nested- polymerase chain reaction (Nested –PCR)

HPV detection and genotyping of the cervical cancer tissue samples were done using the PCR protocol and primer sequences were as described previously (Sotlar et al., 2004). We used Phusion Hot start High Fidelity DNA Polymerase therefore necessary modifications were done for optimal amplification. All PCRs were performed in a final volume of 20 μl . PCR mixtures comprised of 1X Phusion HF buffer, a 200 μM concentration of each deoxynucleoside triphosphate (dNTP), 0.2 U of Phusion Hot start High Fideity Polymerase (Finnzymes, Finland). Genomic DNA 25-50 ng was used for the first round of amplification of a 630bp E6 region using consensus primers (10 μM each). Amplification conditions were as: Initial denaturation 98°C (3 min) followed by 40 cycles of 98°C (15 sec), 54°C (30 sec) and 72°C (45 sec); final extension at 72° C (7 min). Two microlitres of the E6 region PCR product served as template for the second round of amplification with type specific primers (10μM each). Nested Multiplex PCRs (NMPCRs) with type-specific primers were performed under the following conditions: Initial denaturation 98°C (3 min) followed by 35 cycles of 98°C (15 sec), 56°C (30 sec) and 72°C (45 sec); final extension at 72° C (7 min).

The integrity of genomic DNA was verified by the amplification of a 248-bp product of the human beta-globin housekeeping gene. The following primers were used: HMBB01, PC04 and GH20 (5 μM each). Amplification condition were as: Initial denaturation 98°C (3 min) followed by 35 cycles of 98°C (15 sec), 56°C (45 sec) and 72°C (60 sec); final extension at 72° C (10 min).

Fifteen microlitres of the all PCR products were electrophoresed on 2% agarose gels (Amresco, USA), stained with ethidium bromide (Amresco, USA; 0.625mg/ml) and visualized in Gel Documentation system (Bio-Rad, USA). HPV -11, -16, -18, -45, -58 and -59, DNA-containing plasmids (1 ng) were used as positive controls.

Hybrid capture 2 (HC2) assay

Cervical smear from patients were collected in DNAPAP Cervical Sampler kit (Qiagen, Germany) and subsequently Digene HPV Hybrid Capture II Test for human Papillomavirus types was done. Chemiluminescent detection of the 13 most common HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) was performed as per the manufacturer's instructions using the Automated DML 2000 Luminometer system (Digene, USA). The positive cutoff (CO) value was considered the mean of the positive control samples. The HC2 test, which is FDA-USA approved and WHO recommended for clinical detection of HPV, was used to re-evaluate the multiplex PCR results.

Statistical analysis

Fisher exact test was performed to test the statistical significance of HPV detection with clinical and demographic data of the patients. Where appropriate, 95% confidence intervals (CI) were computed and the P value of statistical significant test was set at 0.05. All data analyses were done in the GraphPad Prism software version 6.01.

Results

Between January 2011- June 2012, a total of 107 eligible cervical cancer patients were enrolled. The HPV detection results were recorded for all the 107 cases, 105 (98%) patient samples tested positive for carcinogenic HPV types and 2 (2%) were found negative for presence of any HPV type. Genotyping of the samples revealed the presence of 6 'carcinogenic' HPV types: HPV-16(88%), -18(15%), -31(4%), -45(3%), -59(4%), -58(1%) and one non carcinogenic: HPV-6/11(6%). HPV-16 was found to be the most prevalent genotype, being present in 88% of the cases. The presence of more than one carcinogenic HPV type was found in 19% of the samples (Table 1).

Ethnicity-wise, 84% tribal and 73% non-tribal patients had shown presence of single carcinogenic HPV types, whereas 15% and 18 % of the respective samples had multiple carcinogenic HPV types. In patients of ≥45 years age 79% and in patients of ≤45 years age 83% of the samples had single carcinogenic HPV-type. On the other hand, multiple HPV types were detected in 18% and 17% of the samples of respective age groups.

Among patients who were of ≤18 years at their first pregnancy, 83% had shown positivity for single HPV and 13% for multiple HPV infection. A similar trend, with 78%

and 22% single versus multiple HPV type, was detected in the group of ≥18 years age.

Only 6% of the patients had practiced safe intercourse at average rate of their active sexual life. In contrast 94% of the patients had never practiced safe intercourse.

In the regular and irregular menstruation groups, single HPV type was detected in 73% and 83% of the cases respectively. Multiple HPV types were found in 15% of regular and 23% of the irregular menstruation group. Eighty four percent of the patients had less than primary level of education as compared to 16% having secondary and above. Among the less than primary level of education group, 82% patients had single HPV as compared to 70% in the higher education group. Family history of cancer in first degree of relatives was reported only in 6% of samples. Previous infection history of genital organs were recorded in 77% of the samples, 83% of these samples had single HPV type and 16% had shown multiple HPV types.

Histologically, 89% cases were confirmed squamous cell carcinoma (SCC) and 7% were adenocarcinoma (ADC). 81% SCC cases and 71% ADC cases had single HPV type. While multiple HPV types were detected in 17% SCC and 28% of ADC cases. There were no statistically significant association found between histological grading (P=0.606), patients age (P=1.000), age at first pregnancy (P=0.315), ethnicity (P=1.000), menstrual status (P=0.396), education status (P=0.482), practice of safe intercourse (P=1.000), family history (P=0.345), previous history of infection (P=0.367) with HPV types (Table 2). Tumor stages I-II had shown presence of single HPV type in 89% samples whereas 10% had multiple HPV types. For stages III-IV, single HPV types were detected in 69% samples and multiple were found in 28%. In the present study tumour staging had shown statistically significant association with HPV types infection (P=0.019) (Table 2).

The presence of high risk HPV (13 types) was confirmed by hybrid capture 2 assay. Nested multiplex PCR and HC2 assay results were in concordance for >99% of the samples. Healthy volunteer control (n=22) were also tested by HC2 assay and all except one were found negative.

Table 1. Prevalence of HPV Genotypes

HPV Genotype(s) Detected	Frequency	% of Total
Total number of Samples	107	100
HPV-16	74	69.1
HPV-18	7	6.5
HPV-31	1	0.9
HPV-45	1	0.9
HPV-58	1	0.9
HPV-59	1	0.9
HPV-16 + HPV-6/11	4	3.7
HPV-16 + HPV-18	7	6.5
HPV-16 + HPV-31	2	1.8
HPV-16 + HPV-45	2	1.8
HPV-16 + HPV-59	2	1.8
HPV-59 + HPV-31	1	0.9
HPV-16 + HPV-18 + HPV-6/11	2	1.8
HPV-Negative	2	1.8

Table 2. Association of Carcinogenic HPV with Demographic and Clinical Variables in Cervical Cancer Patients

Variable			No. (%)	Single HPV	Multiple HPV	P value#
Age	≤ 45 (Range:32-45)		41 (38)	34	7	
(Years)	≥45 (Range:46-79)		66 (62)	52	12	1
Ethnicity	Tribal		20 (19)	16	3	
	Non-tribal		87 (81)	69	16	1
Age at first pregnancy						
	≤18 years		53 (49)	44	7	
	>18 years		54 (51)	42	12	0.31
Safe intercourse practices		Yes	07 (6)	6	1	
		No	100 (94)	80	18	1
Menstruation	Regular		30 (28)	22	7	
	Irregular		77 (72)	64	12	0.39
Education	Up to Primary		90 (84)	74	15	
	Secondary and Higher		17 (16)	12	4	0.48
Histological Grade	SCC		95 (89)	77	16	
	ADC		07 (7)	5	2	0.6
Tumor Staging	I-II		61 (57)	54	6	
	III-IV		46 (43)	32	13	0.01*
Family History	Yes		07 (6)	7	0	
	No		100 (94)	79	19	0.34
Previous Infection History of Genital						
	Yes		82 (77)	68	13	
	No		25 (23)	18	6	0.36

*Statistically significant, SCC- Squamous Cell Carcinoma, ADC- Adenocarcinoma, #Fisher's Exact Test

Discussion

We found HPV -16 to be the most prevalent carcinogenic genotype followed by the HPV-18, -31, -45, -58, -59 and non carcinogenic HPV- 6/11. It may be noted that HPV-31, -45, -58,-59 (<1% each) were also detected alone in few of the samples but the majority of these genotype were found along with HPV-16. Though the prevalence of other carcinogenic genotypes is quiet low compared to HPV-16 and -18, still they represent around 5% of the study population. The low risk genotype 6/11 was found in 6% of the samples but always along with HPV-16 and/or 18. Currently without large healthy population screening data it is impossible to suggest which genotype is established a priori.

Our study suggests that patients in advanced tumour stage III-IV had increased risk of single and multiple HPV types as compared to the patients of stage I-II. Most of the patients in Group I-II were diagnosed as IIB whereas in group III-IV was IIIB. At present it is not clear whether infection by multiple HPV types can serve as a predictor for the overall survival of the patients because the follow up period extends to minimum 36 months. However, several studies have provided evidence that multiple HPV infection seems to be associated with a significantly increased risk of high-grade neoplasia as compared with single infection (Cuschieri et al., 2004; Herrero et al., 2005). In India, 85-90% cervical cancer cases are squamous cell carcinoma (SCC) but only 10-15% cases are adenocarcinoma (ADC). A Meta analysis found that the proportion of SCC and ADC in North India was 95.3% and 3.8%, while in South India it was

94.8% and 5.2%, respectively. The HPV 16/18-positive fraction was 78.9% overall with some variation between North and South India (Bhatla et al., 2008). HPV -16 is the most prevalent type both in squamous cell carcinoma as well as adenocarcinoma while global reports indicate preferential occurrence of HPV -18 in adenocarcinoma (Das et al., 1993; Iwasawa et al., 1996; Deodhar et al., 2012). We found 89% squamous cell carcinoma and 7% adenocarcinoma patient samples which suggest that our data shows similar trend as reported by previous studies from other regions of India.

Together, high-risk HPV -16, -18 and low risk HPV -6/11 was confirmed in >92% of the patients. This observation has significance with reference to the currently available HPV vaccines viz. Gardasil™ and Cervarix™. We detected in 88% patients HPV -16 and HPV-18 alone or in combination. A recent multicentre study has shown that 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. 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% (Basu et al., 2009). Another multicentre study with total 18,085 women subjects (Normal, CINI, II, III and invasive cervical cancer) from three cities (Mumbai, Kolkata and Trivandrum), had found HPV positive cervical cancer cases (n=38) as 85.7% and 66.7% in two centres of Kolkata, 76.9% in Trivandrum, 73.7% in Mumbai (Sankaranarayanan et al., 2004). In a previous North East India related study Ghosh et al. (2011) reported that HPV -18 compared to HPV -16 is the prevalent genotype in cervical cancer patients from southern Assam. The disagreement in our findings with Ghosh et al data could be attributed to the variation in patient population, methodology used in the two studies. However our samples represented patient from various parts of Assam and other north-eastern states.

HPV transmission takes place, mainly, by sexual contact and the organs most susceptible to infection with potential of starting a neoplastic transformation are the cervix (transformation zone) and the pectineal line of the anal canal. HPV infections are frequently present in sheet of epithelial cell and HPV DNA can be detected in the cervix, vagina, and vulva in the woman, the glans, prepuce and skin of the penis and scrotum in the man, and in the anal canal and perianal area in women and men (Castellsagué, 2008). Although fewer studies have been conducted on the prevalence of HPV infection among men than among women, HPV infections also appear to be common in men (Gabriella and Anna, 2011).

The high prevalence of HPV -16 (88%), in the studied patient population may be the consequence of unsafe genital hygiene and sexual behavior because a vast majority (94%) of the patients had reported lack of safe sexual behavior throughout their active sexual life. It is also worth to mention here that 77% of the patients or their spouse had a history of previous infection in genital organ. During personal interviews it had been observed that many of the patients were unable to understand what can be defined as infection and failed to seek any medical opinion for their infection related symptoms. This lack of awareness may have root in the fact that 84% of the patients were educated less than primary level. It is well recognized that cervical cancer risk is associated with a low socio-economic status, as defined by education or income levels. The reasons for the association are not fully understood but are thought to include a higher prevalence of cervical cancer risk factors, such as inadequate cervical cancer screening, high parity and possibly high-risk sexual behaviour among women with a low socio-economic status (Franeschi et al., 2009). The tribal patient numbers (n=20) were quiet less compared to the non-tribals (n=87), but HPV prevalence and distribution had not shown any significant difference between the two group. Fewer tribal patients turnout may be attributed to the fact that mostly tribal populations reside in difficult terrain areas and have little access to modern healthcare facilities in their near vicinity.

The histological diagnosis was found to be predominantly squamous cell carcinoma. It had also been noticed that a majority of the patients presented themselves to the Gynaecologic Oncology department after many months since appearance of any possible clinical symptom of cervical cancer. This could be the possible reason that the disease progressed to an advanced stage.

We were unable to collect detailed information about sexual behaviour, number of sexual partners of women, and the sexual history of women's male partner because of the socio-cultural customs prevailing in the population.

Knowledge of the prevalence and natural history of type specific HPV, either as single or multiple infections, in the development of cervical neoplasia will be important for an appropriate intervention in cervical cancer prevention programme. The major steps known to be necessary for cervical cancer development include HPV infection, persistence of that infection, progression to precancerous lesions and eventually invasion. Provided that the latter step has not taken place, this process is reversible by the clearance of HPV infection and regression of pre-cancer. Other than HPV DNA testing, awareness about safe sexual behaviour, early clinical symptoms of cervical cancer and improvement of socio-economic status may also have important role in reduction of cervical cancer cases.

In conclusion, the findings presented in this study describe the HPV type distribution among women diagnosed with cervical cancer. Altogether seven genotypes were detected by nested multiplex PCR: HPV 16, 18, 6/11, 31, 59, 58 and 45. HPV -16 was found to be the most prevalent genotype in the north east cervical cancer patient population. Patients being a part of the community with similar life styles and exposure to risk factors, the findings on the genotype distributions in cervical cancer patients may also provide some insight about probable HPV types harboured in the apparently healthy members of the community. However, further studies in asymptomatic adolescent and young women may be necessary to ascertain the diverse HPV types present in north East Indian population.

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References

Basu P, Roychowdhury S, Bafna UD, et al (2009). Human papillomavirus genotype distribution in cervical cancer in india: results from a multi-center study. *Asian Pac J Cancer Prev*, **10**, 27-34

Bhatla N, Lal N, Bao YP, Ng T, Qiao YL (2008). A meta-analysis of human papillomavirus type-distribution in women from South Asia: Implications for vaccination. *Vaccine*, **26**, 2811-7.

Bosch FX, Manos MM, Munoz N, et al (1995). Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. *J National Cancer Institute*, **87**, 796-802.

Bouvard V, Baan R, Straif K, et al (2009). A review of human carcinogens—part B: biological agents. *Lancet Oncol*, **10**, 321-2.

Broomall EM, Reynolds SM, Jacobson RM (2010). Epidemiology, clinical manifestations, and recent advances in vaccination against human papillomavirus. *Postgraduate Med*, **122**, 121-9.

Burchell AN, Winer RL, Sanjos´e SD, Franco EL (2006). Epidemiology and transmission dynamics of genital HPV infection. *Vaccine*, **24**, 52-61

Castellsagué X (2008). Natural history and epidemiology of HPV infection and cervical cancer. *Gynecol Oncol*, **110**, 4-7.

Cuschieri KS, Cubie HA, Whitley MW, et al (2004). Multiple high risk HPV infections are common in cervical neoplasia and young women in a cervical screening population. *J Clinical Pathol*, **57**, 68-72.

Das BC, Gopalkrishna V, Das DK, et al (1993). Human papillomavirus DNA sequences in adenocarcinoma of the uterine cervix in Indian women. *Cancer,* **72**, 147-53.

Deodhar K, Gheit T, Vaccarella S, et al (2012). Prevalence of human papillomavirus types in cervical lesions from women in rural Western India. *J Med Virology*, **84**, 1054-60.

Ferlay J, Shin HR, Bray F, et al (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer*, **127**, 2893-917.

Franceschi S, Plummer M, Clifford G, et al (2009). Differences in the risk of cervical cancer and human papillomavirus infection by education level. *Br J Cancer*, **101**, 865-70.

Gabriella MA, Anna RG (2011). Genital HPV infection and related lesions in men. *Prev Med*, **53**, 36-41.

Ghosh SK, Choudhury B, Hansa J, et al (2011). Human papillomavirus testing for suspected cervical cancer patients from southern assam by fast-PCR. *Asia Pac J Cancer Prev*, **12**, 749-51.

Gopalkrishna V, Hedau S, Kailash U, Das BC (2000). Human papillomavirus type 16 in cancer of the uterine cervix in different geographical regions of India. International HPV conference 2000. Abstract, 077.

Herrero R, Castle PE, Schiffman M, et al (2005). Epidemiologic profile of type-specific human papillomavirus infection and cervical neoplasia in Guanacaste, Costa Rica. *J Infectious Disease*, **191**, 1796-807.

Iwasawa A, Nieminen P, Lehtinen M, Paavonen J (1996). Human papillomavirus DNA in uterine cervix squamous cell carcinoma and adenocarcinoma detected by polymerase chain reaction. *Cancer*, **77**, 2275-79.

Munoz N, Castellsagué X, de Gonzalez AB, Gissmann L (2006). HPV in the etiology of human cancer. *Vaccine*, **24**, 1-10.

Pista A, Oliveira A, Verdasca N, Ribeiro F (2010). Single and multiple human papillomavirus infections in cervical abnormalities in Portuguese women. *Clin Microbiol Infection*, **17**, 941-6.

Raychaudhuri S, Mandal S (2012). Current status of knowledge, attitude and practice (KAP) and screening for cervical cancer in countries at different levels of development. *Asian Pac J Cancer Prev*, **13**, 4221-7.

Sankaranarayanan R, Chatterji R, Shastri SS, et al (2004). Accuracy of human papillomavirus testing in primary screening of cervical neoplasia: results from a multicenter study in India. *Int J Cancer*, **112**, 341-7

Schiffman M, Wentzensen N, Wacholder S, et al (2011). Human papillomavirus testing in the prevention of cervical cancer. *J Natl Cancer Inst*, **103**, 368-83.

Smith JS, Lindsay L, Hoots B, et al (2007). Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. *Int J Cancer*, **121**, 621-32.

Sotlar K, Diemer D, Dethleffs A, et al (2004). Detection and typing of human papillomavirus by E6 nested multiplex PCR. *J Clinical Microbiol*, **42**, 3176-84.

Thulaseedharan JV, Malila N, Hakama M, et al (2012). Socio-demographic and reproductive risk factors for cervical cancer – a large prospective cohort study from rural India. *Asia Pac J Cancer Prev*, **13**, 2991-5.