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Research Article

Oncogenic Human Papilloma Virus type/s Detection in Cytologically Abnormal Females – Clinical Relevance for Disease Management

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Abstract: A precise subset of Human Papillomavirus (HPV) genotypes, called high-risk genotypes, has now been undeniably established as the cause of invasive cervical cancer. HPV16 and 18 account for 70% to 80% of cervical cancers. Present study was planned to detect and type the High risk HPV in cytologically abnormal women. 80 cervical brushings were collected and subjected for pap smear followed by Polymerase chain reaction and genotyping. 24(30%) cases came positive for high risk human papillomavirus. Pap abnormal cases, categorized as ASCUS, LSIL & HSIL were studied for presence of HR-HPV genotypes. 06, 27, 25, & 22 specimens were respectively in ASCUS, LSIL, HSIL & other cervical abnormalities categories out of which 01(0.16%), 07(25.9%), 12(48.0%), and 04(18.1%) were HR-HPV positive. Genotyping of HPV revealed that HPV type 16 with 10(41.6%) cases was the most prevalent type detected followed by HPV type 18 with 06(25.0%) cases and 03(12.5%), 03(12.5%) and 02(8.3%) cases respectively of HPV 16 and 18, HPV 31 and other HR-HPVs. Mixed infection of HPV type 16 and HPV type 18 was seen in 03 cases. HPV prevalence Genotypic characterization with respect to cytological status of the cervix is of clinical relevance for proper guidance screening and subsequent follow up of the patiets as well as provide the ideas for strategic evaluation to vaccine production & research to design the related therapeutics.
Keywords: High risk Human Papilloma Viruses, Pap Smears, Cervical cancer, Polymerase Chain Reaction, Neoplasia.

INTRODUCTION:

Cervical cancer is the second most common cancer in women worldwide, however 80% of the cases are suspected from developing nations [1]. The human papillomavirus (*HPV*) is a well-known cause of cervical cancer in women [2]. Carcinoma of the uterine cervix is the most common cancer in Indian women. To an estimated annual global incidence of 500,000 cervical cancers, India contributes 100,000, i.e. 1/5 of the world burden. Knowing a patient's HPV type/s provides clinicians with valuable information to guide screening, treatment and subsequent follow-up [3]. Persistent infection with High-risk (HR) HPV genotypes ultimately has the potential to cause carcinogenesis of the cervix. High-risk types 16 and 18 have been reported to cause 70% of cervical cancers and 90% of the head and neck cancers caused by HPV [4]. Thus HPV genotypic identification of these genotypes will help in close monitoring of patients and prevention of cervical cancer [5]. Cervical cancer involves cells of the cervix which will become irregular and proliferate results in formation of tumors [6]. More than 40 types of Human Papillomavirus (HPV) are acknowledged to infect the genital tract. Fifteen are classified as high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), and 12 as low-risk (6,11,40,42,43,44,54,61,70,72,81, and CP6108), but still some of the low risk HPV types may cause cancer [7]. Most of the HPV infections clear up on their own; the infections could increase to major abnormalities and can lead to cervical cancer [8, 9].The clinically significant strains 16, 18 and 31are the prime risk factors for cervical cancer. Walboomers *et al.;* reported that the existence of HPV is a necessary situation for the development of cervical cancer. A virus cancer link with HPV has been found to trigger alterations in the cells of the cervix, leading to the development of cervical intraepithelial neoplasia and cancer. The extensive introduction of the Papanicolaou test or Pap smear for cervical cancer screening has been attributed with noticeably reducing the incidence and mortality of cervical cancer in developed countries [10]. The Pap smear suggests the presence of cervical intraepithelial neoplasia before cancer develops, allowing for further

follow-up. Cervical cancer screening is presently done by finding abnormal cells in cervical smears (i.e., cervical cytology or Pap smears). Cervical cytology is insensitive for the detection of cancer and pre-cancer requiring many rounds of screening to achieve programmatic effectiveness. The advent of Nucleic Acid Amplification Techniques (NAATs) and other molecular diagnostics tools, have markedly increased essential parameters like sensitivity and specificity for the detection of HPV [11]. Detection of HPV genomes and transcripts can be achieved with hybridization procedures including Southern and Northern blots, dot blots, in-situ hybridization, signal-amplification molecular technology (Hybrid Capture assay, version hc2; Digene, Gaithersburg, MD, USA), and DNA sequencing [12,13]. For proper treatment, follow-up and launching vaccination programmes, type specific HPV detection is very significant. There are several findings which showed a high frequency of multiple HPV infections in cervical carcinomas [14]. Regardless of the elevated incidences of cervical cancer reported from India, large-scale population based studies on the HPV prevalence and genotype distribution are very few [15]. Thus the present study was done to detect and type the HPV genotype/s in cytologically abnormal females and its correlation with Pap smears.

MATERIALS AND METHODS

A total of 80 cervical brushings were collected from Gynecology and Obstetrics Department of Shri Mahant Indiresh Hospital, Dehradun, Uttarakhand. The inclusion criterion for the selection of the cases were women with cervical erosion, lower abdominal pain, postcoital and intermenstrual bleeding, menstrual irregularities, pelvic inflammatory disease (PID) etc. The study was approved by ethical clearance body and written consent from patients was taken. The cases for the proposed study includes the females with abnormal pap smear results which includes; Atypical squamous cells of undetermined significance (ASCUS), High grade squamous epithelial lesion (HSIL), Low grade squamous intraepithelial lesion (LSIS) and females with other cervical complications. All the specimens were subjected for DNA isolation and Polymerase chain reaction (PCR). The DNA was isolated from every cervical brushing specimen by Magnetic beads method (MagDEA lab viral DNA\RNA 200, Germany) [16]. Further the isolated DNA was utilized for High Risk HPV (HR-HPV) PCR method targeting early genes E6 and E7. [17]. The PCR was carried out in 0.2 ml PCR tube in Applied Biosystem veriti Thermalcycler. For a single reaction add 21µl of HPV master mix and 0.4µl of Hot start Taq DNA polymerase (as per the manufacturer's instructions). Further 3µl template DNA was added to the master mix and subjected for HPV PCR. Cycling conditions includes; Initial denaturation at 94°C, for 5min, with 10 repetitive cycles of denaturation for 94°C,anneling at 62°C and extension at 72°C for 60 seconds, followed by 30 repetitive cycles each for 45 seconds of denaturation at 94°C, annealing at 58°C extension at 72°C. Further final extension was given at 72°C for 5min. Amplicons were visualized in 1.6% agarose gel, stained with ethidium bromide, yielded an amplicon product of 233 bp for high risk human papilloma virus. For detection of the type specificity of HPV, further the amplicons were digested with restriction enzymes and different genotypes yielded different restriction fragments products in agarose gel electrophoresis.

RESULTS

Total of 80 cervical brushing specimens from females were considered for the study, out of which 24(30%) cases came positive for high risk human papillomavirus (as tabulated in table 01). It was also seen that the HR-HPV positive cases were found in age group 21-40 and 41-60 (as tabulated in table 01). Specimens were categorized into four categories, as per the clinical manifestations and PAP smear results as; ASCUS, LSIL , HSIL and other cervical abnormalities (bleeds on touching cervical erosion, PID, etc.) 06, 27, 25, & 22 specimens were respectively in ASCUS, LSIL, HSIL & other cervical abnormalities categories out of which 01(0.16%), 07(25.9%), 12(48.0%), and 04(18.1%) were HR-HPV positive cases in these groups (Table 2). Type specific HPV were detected by digesting amplicon with restriction enzymes (as shown in figure 2). HPV type 16 with 10(41.6%) cases was the most prevalent type detected followed by HPV type 18 with 06(25.0%) cases and 03(12.5%), 03(12.5%) and 02(8.3%) cases respectively of HPV 16 and 18, HPV 31 and other HR-HPV s (Table3). Mixed infection of HPV type 16 and HPV type 18 was seen in 03 cases.

Table 1. Age wise distribution of high risk human papilloma virus (Hr-HPV)

Age in years	Total cases	Hr-HPV positive	Hr-HPV negative	Genotype/s Detected
0-20	01	00 (0%)	01	None
21-40	38	12 (31.5%)	26	HPV type 16, 18, 31 and other HR-HPV's
41-60	30	10(33.3%)	20	
Above 60	11	02 (18.1)	09	
Total = 80		**24 (30.0%)**	**56**	

Table 2. Genotypic distribution of HPV in cytologically abnormal PAP and symptomatic females

Total specimens	PAP smear results	No. of specimens	High risk HPV positive cases	HPV type/s detected
80	ASCUS[1]	06	01 (0.16%)	HPV -16
	LSIL[2]	27	07 (25.9 %)	HPV type 16, 18, 31 and other HR-HPV's
	HSIL[3]	25	12 (48.0 %)	
	Other cervical abnormalities	22	04 (18.1%)	

[1]Atypical squamous cells of undetermined significance
[2]Low grade squamous intralepithelial lesions
[3]High grade squamous intralepithelial lesions

Table 3: High risk HPV type/s distribution in high risk human papilloma virus (Hr-HPV)

Total HR-HPV Positive= 24					
Different HPV type/s	HPV-16	HPV-18	HPV 16& 18	HPV-31	Other HR-HPVs
Positive cases for specific type/s	10 (41.6%)	06 (25.0%)	03 (12.5%)	03 (12.5%)	02 (8.3%)

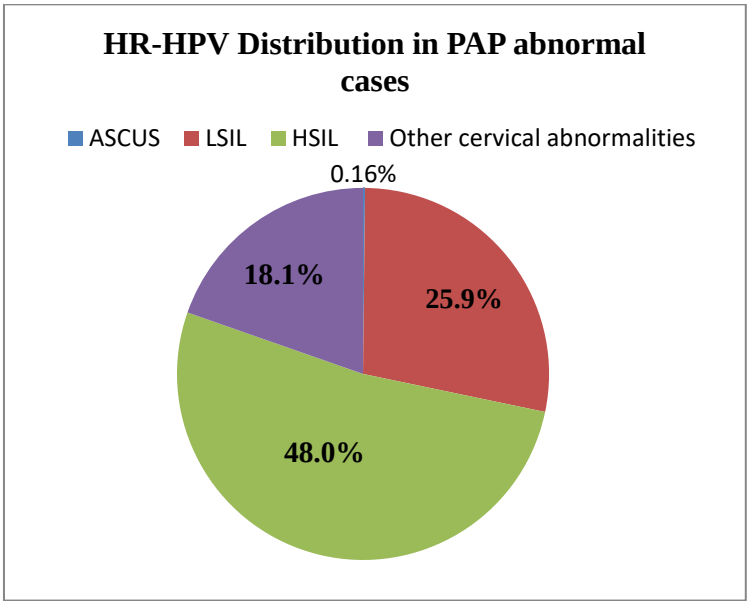

Fig.1. Pie chart depicting the high risk HPV distribution in abnormal PAP smears cases.

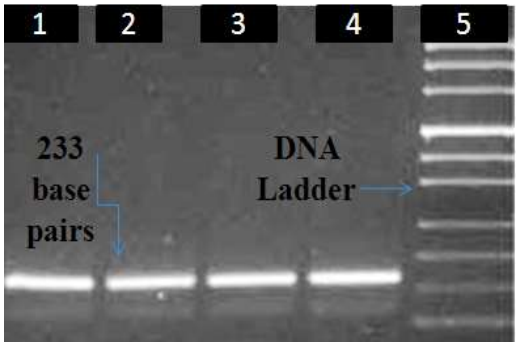

Fig 2. Gel picture for HPV positive product at 233 bp

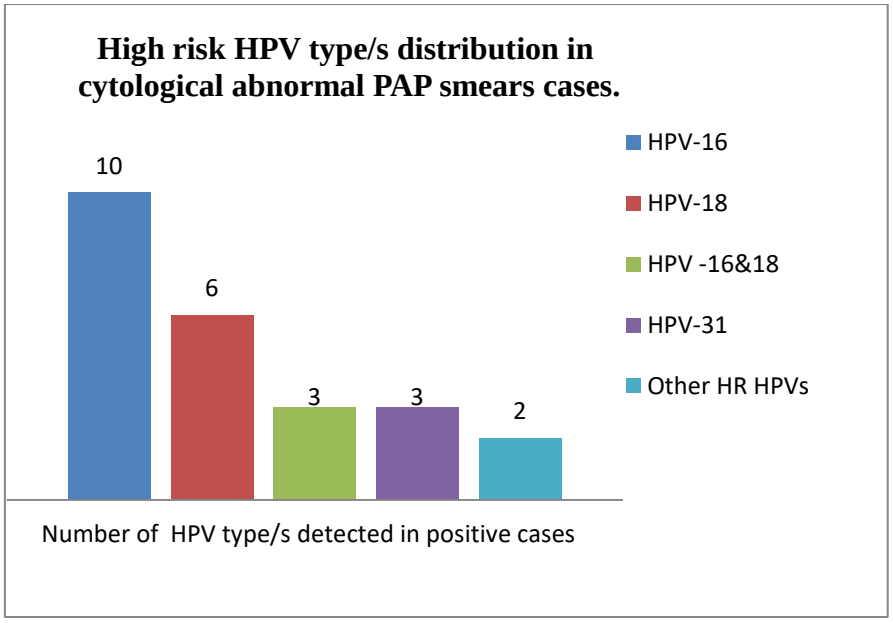

Fig.-2: Chart depicting the different HPVs distribution in HR-HPV positive cases.

DISCUSSION AND CONCLUSION

Worldwide, HPV16 and 18 (the two vaccine-preventable types) contribute to over 70% of all cervical cancer cases, between 41% and 67% of high-grade cervical lesions and 16-32% of low-grade cervical lesions. After HPV16/18, the six most common HPV types are the same in all world regions, namely 31, 33, 35, 45, 52 and 58; these account for an additional 20% of cervical cancers worldwide. About 67,477 new cervical cancer deaths occur annually in India (estimations for 2012) and is the 2nd leading cause of cancer deaths in women aged 15 to 44 years in India [18]. Cervical cancer causes loss of productive life both due to early death as well as prolonged disability. In India, the Years of Life Lost (YLL) due to cervical cancer were 936.3 in 2000, being among the highest in the world, greater than the YYLs caused by any other cancer in India constituting almost 4% of total YYLs due to all causes in India [18]. Females with associated cervical complications in developing nations are mostly not from sound background, even refuse or can't afford for significant DNA based HPV testing. In the current study, Molecular characterization of oncogenic HPVs from females of Uttarakhand population will also provide innovative ideas for the strategic evaluation to pharmaceutical industries, vaccine production and research and development industries to design the drugs and related vaccines for this area. The outcome or the findings of molecular diagnostics assay for HPV is very important for further management as well as for proper follow up of this disease. Women with HPV result negative can safely return to Pap smear routine screening and their risk of cancer is negligible. Only women with HPV positive result require instant reference for colposcopy. Women with an equivocal Pap smear or low grade cervical dysplasia are advised to have HPV test. Those women having HPV result negative can be safely return to Pap smear routine screening their risk of cancer is negligible [12]. The women with High Grade Squamous Intra Epithilial Lesion (H.G.S.I.L.) have more chances of HPV infection. In our study most of the HPV positive cases showed the presence of genotype HPV16 and 18 but there is also the cases for high risk HPV type 31 which was present in 03 cases and about 20.8% (05 cases) harbor Hr- HPV type 31 and other Hr-HPVs like 33,35,45,52 and 58 which are clinically relevant for the disease progression and invasive cancer. HPV type 6 and 11 are less harmful & these genotypes causes' genital warts & mostly do not cause the cancer [13]. With the emergence of HPV vaccines and large-scale clinical trials proposed and under way, awareness in the distribution of HPV genotypes prevalent in the Indian population has gained clinical significance. HPV vaccines Gardasil (Merck) and Cervarix (GSK) predominantly target HPV type 16 and 18. These vaccines may prove to be effective against a majority of cervical cancers, but may not benefit women infected with rarer HPV types [14, 15]. Primary approaches to prevent HPV infection include both risk reduction and development of HPV vaccines. Another challenge for programs that ultimately offer an HPV vaccine is how to "position" and promote the vaccine. Should it be described as an "anti-cancer" vaccine (which would appeal primarily to women) or an anti-STD vaccine (which raises difficult social issues in most cultures) or even an anti-wart vaccine (which may broaden the vaccine's appeal, particularly in young, sexually-active populations).These questions are important ones; planning how to promote the vaccine would have a major impact on its ultimate success or failure. Some researchers have promoted the development of regional

vaccine formulations that are tailored to prevent locally prevalent types of HPV. More epidemiological research on the prevalence of various HPV types is required before the need for regionally-tailored vaccines is confirmed, however. Thus the use of several genotyping methods like conventional PCR is very helpful as it can detect different genotypes of HPV so that the existing vaccines for the particular genotype can be prescribed as well as for HPV types causing generalized and genital warts, proper treatment can be given to the patient. Several studies have now shown that detection of specific carcinogenic HPV types, especially HPV type 16 (HPV16) and HPV18 may be useful in differentiating carcinogenic HPV-positive women at greater and lower risk of having or developing precancer, cervical intraepithelial neoplasia grade 3 (CIN3), or cancer (CIN3+). Identifying women with persistent carcinogenic HPV infection is clinically useful. It is thus of great importance to assess if there are significant differences in the geographic distribution of HPV types causing cervical cancer. If this is the case, their description would provide the rationale for considering tailoring vaccine products to the HPV types prevalent in a given geographic area vs. producing a common vaccine suitable to the worldwide distribution of HPV types.

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