ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 7, Suppl 2 2014

ISSN - 0974-2441

Research Article

CLONING AND CHARACTERIZATION OF HIGH RISK HUMAN PAPILLOMA VIRUS (HPV) ONCOGENE E6.

KARRAR ABDULAMEER JUMAAH, SUDHAKAR MALLA, R.SENTHIL KUMAR

Centre for Research & PG studies, Indian Academy Degree College, Bangalore India.

Email: karrarabdulameer2013@yahoo.com

Received: 3 March 2014, Revised and Accepted: 3 April 2014

ABSTRACT

Cervical cancer has always been a burden for developing countries which suffer with considerable barriers to set up cytology-based screening programs. HPVs, also called human papilloma viruses, are a group of more than 150 related viruses. More than 40 of these viruses can be easily spread through direct skin-to-skin contact during vaginal, anal, and oral sex. HPV had been proved to play a crucial role in the development of cervical cancer. HPV mainly targets the tumor suppressor proteins via the oncogenes E6 and E7. These alter the host cell metabolism and prevents the cell from apoptosis. Viral based vaccines are very few for the HPV diagnosis. Novel methods are being developed for the production of antibodies to specific antigens and thus helping in the process of development of protein based vaccines.

E6 gene was isolated and ligated into pTZ57R/T cloning vector. The ligated product was then cloned into DH5α strain and allowed to propagate. The plasmids thus cloned were purified and later expressed for the gene of interest in an expression vector. The proteins specific to the gene of interest was then isolated and purified. This proteins purified can in turn be used for protein based vaccines.

Keywords: HPV, E6 gene, p53, oncogenes.

INTRODUCTION

Cervical cancer and its relation with HPV virus had already been established and so far many diagnostics based on cytology have been developed(Parkin DM;2002). Molecular diagnostics are on the rise, and many genes which are proved to play a critical role in pathogenesis has been traced out (Arens M et al;2001). Cervical cancer has always been a burden for developing countries which suffer with considerable barriers to set up cytology-based screening programs(Parkin DM;2006). Moreover high-quality cytology requires trained personnel and sophisticated equipment (Hariri S et al;2011, Anna-Barbara Moscicki et al; 2010). The establishment of HPV as central and necessary cause of cervical cancer was exploited for the development of molecular diagnostics and to overcome limitations linked to cytologic cervical screening.

In cervical cells, HPV occurs either in episomal form or integrated form or sometimes both(Neerja Bhatla et al; 2009, Hamid NA et al; 2010). Research suggests that identification of integrated viral forms could support HPV based screening and triage strategies. As HPV cannot be grown in conventional cell cultures, and at the same time serological assays also have only limited accuracy (Dillner, 1999).

Human papillomavirus (pap-ah-LO-mah-VYE-rus) (HPV) is the most common sexually transmitted virus in the United States. Almost every sexually active person will acquire HPV at some point in their lives. Human papillomavirus (HPV) is a significant source of morbidity and mortality worldwide (Arens M et al, 2001; Beasley RP et al;1977).

HPVs, also called human papilloma viruses, are a group of more than 150 related viruses. More than 40 of these viruses can be easily spread through direct skin-to-skin contact during vaginal, anal, and oral sex (Arends MJ, Donaldson YK, Duval E, *et al*, 1993). HPV is a small DNA virus with a genome of approximately 8000 base pairs. Scheurer ME, 2005).

HPV targets the basal cells in the stratified squamous epithelium and the metaplastic cells at the squamocolumnar junction of the cervix. Moreover, HPV infects the glandular epithelium of the endocervix and thus leads to what is called as glandular neoplasm (Lilienfeld AM;1959).

The two primary oncogenes of high-risk HPV types are E6 and E7 (e stands for early in the HPV life cycle). These two genes encode for proteins which alter the host cell metabolism which in turn favours the development of neoplasticity(Munger K et al; 1992). Usually E6 binds to and degrades the host-cell protein p53. This is an adaptive mechanism to prevent the apoptosis of the infected host epithelial cells(Gissmann L et al; 1993). During this process telomerase is also activated, which further augments the oncogenic changes(Jacobs MV;2000). The E7 protein also has a similar effect on host cell metabolism, which binds to the retinoblastoma protein, inhibiting its function. This leads to disruption of the cell cycle (Scheffner M et al; 1991, Tindle RW, 2001). In addition, E6 and E7 proteins may also cause chromosomal destabilization, and inhibits the cyclin-dependent kinase inhibitors (zur Hausen H; 2000).

The HPV genome contains several genes that encode proteins. In the HPV genome, three of these genes E2, E6, and E7 are of particular interest (zur Hausen H et al; 2001, Francheschi S et al; 2003). They play a crucial role in the development of cervical cancer. The E2 protein binds to both the E6 and E7 proteins (Winer RL et al; 2003, Bosch FX et al;2002). When E6 and E7 are bound to E2, they are blocked from their normal actions in the cell. Once the HPV integrates into the host's DNA, the viral genes E6 and E7 may be over-expressed. This over expression leads to the formation of proteins which prevent the activity of tumor suppressors. Here, E6 can inhibit p53, a protein that controls responses to different types of cellular stress, including DNA damage and viral infection(Arens M et al; 2001).

The present study is mostly focused to isolate the DNA load from cervical tissue samples infected by viral cancer. The study mostly concentrated on isolating the E6 gene. The gene was purified, amplified and cloned into a suitable vector. The genes are then isolated and sequenced and expression analysis was done on SDS PAGE for the viral oncogene E6. This method of study might be used for vaccine production against the E6 antigen.

MATERIALS AND METHODS

Lysate of the HPV culture was kindly donated by Credora life sciences laboratory, Bangalore and was then used for DNA extraction and amplification studies. DNA isolation kit was purchased from the life technologies, PureLink® Viral DNA Mini Kit. Restriction enzymes, dNTPs, Taq polymerase were all purchased from HIMEDIA, India. Oligonucleotides were provided by the Eurofins ltd, Bangalore and were synthesized by the phosphotriester method.

DNA isolation

The protocol was followed according to the manuals instructions from the kit. The viral lysate obtained from the lab, was then used for the extraction process. The viral lysate was added to the spin column, and spin down for 6800g, 1 minute. The columns were then washed down followed by one more centrifugation at 6800g, for 1 minute. After three successive wash steps, the column was again subjected to spin. Then the column was placed in a recovery tube and then eluted with solution E3, which is RNase free. The isolated DNA was then stored at –80°C until further use. The quantity of the isolated DNA was checked in UV-VIS spectrophotomer (Vivaspec Biophotometer, Germany). From the stock 1µl DNA was mixed with 49-µl sterile distilled water to get 50 times dilution. The A260/A280 ratio was recorded to check the purity of DNA preparation.

PCR amplification

The E6 gene was amplified by PCR using purified genomic DNA as a template. Oligonucleotide primers were synthesized to amplify the intact region of E6 gene. The forward primer for E6, 5′ AAACGTTCTGCTCCATCTGC 3′ and the reverse primer, 5′-ATGTGCTGCCCAACCTATTT 3′, were purchased from Eurofins, Bangalore. These primers correspond to the gene E6 and thus the final PCR product was 781bp. The PCR mixture consisted of 10x reaction buffer with $MgCl_2$ (1.5mM), 2µL of dNTP mix (2.5mM), 2 µL each of forward and reverse primers (10picomoles/µl each primer), 0.3µL of Taq DNA polymerase (5 U/µL), and 50ng/ µL of template DNA in a total volume of 20 µL.

The PCR was performed with the following cycling profile: initial denaturation at 94°C for 2 min, followed by 30 cycles of 50s denaturation at 94°C, annealing at 51°C for 30s, and extension at 72°C for 1min. The time for the final extension step was increased to 6 min. The PCR products amplified were then qualitatively analysed on 1% agarose gel. The PCR product was recovered using the QIA quick gel extraction kit, and the amplified product was then purified and used for cloning purpose.

Primer Details

Table 1: Table showing the details of the forward and reverse primers designed towards amplification of the E6 gene of HPV.

Primer	Sequences (5'- 3')	GC %	Tm Value	Length	Product Size
FW	AAACGTTCTGCTCCATCTGC	50	60.4^{0}C	20	781bp
RV	ATGTGCTGCCCAACCTATTT	45	59.5^{0}C	20	

Cloning of the *E6* gene

The purified PCR product was ligated into the pTZ57R/T cloning vector (Fermentas, USA) and the resulting plasmid was transformed into the competent *E. coli* JM109. A 30 µl ligation reaction was setup in 3:1 molar ratio of insert and vector DNA as follows. 6µl of PCR product (0.52pmol) was ligated with T4 DNA ligase (5U) and vector of concentration 50ng/µl was used. The total volume of the reaction mixture was made up to 30µl with nuclease free water (Sigma Aldrich). Ligation mixture was incubated at room temperature (25°C) for one hour after a short spin. The ligated product was later kept on ice until the transformation experiment started.

Competent cell preparation and transformation

The competent cells were prepared using the protocol as described in Molecular cloning (Sambrook and Russel, 1989). *E. coli* DH-5-α was inoculated into 5ml of Luria Bertani (LB) broth. The culture was incubated overnight at 37°C in an orbital shaker at 200rpm and allowed to grow until the optical density reached about 0.375(A600). Then the cultures were transferred to pre-chilled centrifuged tubes followed incubation for 10 minutes on ice and later subjected to calcium chloride treatment (0.1M $CaCl_2$) and made into aliquots of 200 µl and stored for further use at -80°C in 40% glycerol. The aliquots are used for transformation.

The ligated product was mixed with 200µl of prepared competent cells and incubated on ice for 30 minutes without disturbing followed by heat shock treatment at 42°C for 2 minutes. The tubes were then incubated on ice for 2minutes. To the treated cells 1ml of LB broth was added and the tubes were incubated in an orbital shaker at 37°C for 1 hour with an agitation of ~200rpm. During the incubation period, 50ml of LB agar was melted and allowed to cool to 40°C. To the 50ml of molten LB agar, 50 µl of Ampicillin (40mg/ml) to a final concentration of 40µg/ml, 200µl of X-Gal to a final concentration of 80µg/ml and 20µl of IPTG to a final concentration of 80µg/ml was added. The incubated culture after 1 hour was centrifuged at 1000rpm for 10 minutes at room temperature and the pellet was resuspended in 100 µl of fresh LB broth. From the suspension, 100 µl was spread on LB agar plate. The plates were then incubated at 37°C overnight.

White colonies containing recombinant plasmids due to the insertional inactivation of the *lacZ* gene were selected and streaked on a fresh LB plate containing ampicillin and incubated overnight and served as a master plate for each tranformant. All colonies from the master plate were subjected to plasmid DNA isolation and restriction analysis to identify the positive recombinants.

Plasmid isolation by alkaline lysis method

The selected colonies were inoculated separately into 2ml of LB medium containing Ampicillin (50µg/ml) in a 15ml sterile tube. The cultures were incubated overnight at 37°C with vigorous shaking. 1.5ml of overnight culture was centrifuged at 12000 rpm for 30 seconds at 4°C in a microcentrifuge. The bacterial pellet was resuspended in 100 µl of ice cold solution I (50mM glucose, 25mM Tris-Cl and 10mM EDTA; pH 8.0) by vigorous vortexing. To the suspension 200µl of freshly prepared solution II (0.2N NaOH, 1% w/v SDS) was added and mixed properly. The contents of the tube were mixed by inverting the tube rapidly for five times and 150µl of ice cold solution III (5M Potassium acetate, 12.5 ml glacial acetic acid and the resultant solution made upto 100 ml) was added. The tubes were then vortexed gently and the tubes were incubated on ice for 3-5 minutes and later centrifuged at 12000rpm for 5 minutes at 4°C. The supernatant collected was extracted with an equal volume of chloroform and isoamyl alchohol (24:1). The Plasmid DNA was precipitated with 0.6 volumes of ice cold isopropanol and the DNA was pelleted at 12000rpm and the pellet was washed with 70% ethanol.

Confirmation of clones by restriction digestion

The purified plasmid was subjected to restriction digestion using restriction endonucleases (Merck, India). Restriction digestion was performed in 20 µl reaction volumes with recommended units of enzyme and appropriate buffers at 37°C for 2hours. The plasmid DNA (0.2µg/µl) was double digested with restriction enzyme *Bam*HI (10U/µl) and *Eco* RI (10U/µl). The digested samples were resolved on 1% low melting agarose gel and the released gene of insert was eluted from the agarose gel using gel extraction kit (Bioline USA).

Sequencing

The insert in the purified plasmid was sequenced using ABI PRISM Big Dye Terminators v1.1 cycle sequencing kit (Applied Biosystems Foster city, CA, USA) according to the manufacturer's instructions using T7/M13 primers. The comparison of the nucleotide sequences of the unique fragment with the sequences available in the GenBank database was carried out using the NCBI BLAST program (http//www.ncbi.nlm.nih.gov/blast)

Gene cloning into expression vector

The gene of insert was eluted from the agarose gel using gel extraction kit (Bioline USA). Eluted gene of insert was quantified and ligated with the expression vector pET20b. The reaction mixture was incubated at 27 ^{0}C for 3-4 hours. Vector pET20b (50ng/µl) together with eluted gene of insert(0.52pmole) was added to the ligation mixture and then incubated.

Ligated plasmid was transformed in to the bacterium DH5α as described earlier in this paper and plated on LB medium containing ampicillin (40mg/ml) to a final concentration of 40µg/ml. The white colonies from the LB- Ampicillin agar plates were inoculated in LB-Ampicillin broth and incubated at 37°C for overnight and plasmid DNA was isolated by Alkaline lysis method as described earlier in this paper. The transformed bacteria were inoculated in 50ml of LB broth containing IPTG (0.5mM) as inducer for the expression of the gene. *E.coli* strain without plasmid was used as control. The inoculated culture flask was incubated at 37°C in an orbital shaker at 150 rpm for 24 hours.

Protein extraction

The bacterial cells were harvested by centrifugation (7,000 x g) at 4°C and washed with sterile distilled water. The cells were then suspended in 10 ml of ice-cold acetone (analytical grade), and allowed to stand on ice for 5 min. The suspension after centrifugation (7,000 x g) at 4°C. was air dryed, and the proteins were then extracted by incubating with 1.0 ml of 1% sodium dodecyl sulfate (SDS) for 2 min.The collected protein fractions in sample buffer (10% SDS, 10mM Dithiothreitol, 20% Glycerol, 0.2M Tris-HCl and 0.05% Bromophenol blue) were then separated on SDS PAGE electrophoresis containing 5% stacking gel and 12% resolving gel. The molecular weight of protein bands were determined by comparing them with the molecular weight markers.

RESULTS AND DISCUSSION

Genomic DNA isolation and quantification

The protocol was followed according to the manuals instructions from the kit. The viral lysate obtained from the lab, was then used for the extraction process.The isolated DNA was electrophorized in 1% Agarose gel. The quantity and quality of DNA was analyzed by UV spectrophotometer.

PCR amplification of the *E6* gene

Species specific primers were designed for the HPV using the sequences of E6 gene available in NCBI GenBank using Primer 3 Software. The predicted primers were validated initially *insilico* and subsequently on the thermocycler. The primers could yield an amplicon of the expected size specific E6 gene. The primers were found to produce ~781bp amplicon which shown in the figure1.

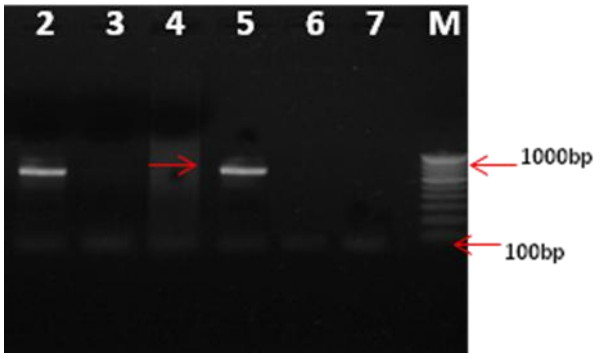

Fig.1: PCR amplification of E6 gene by specific primers (M- 1kb ladder, Lane 2, 5- E6 gene product, 3, 4, 6 & 7- Negative control)

Cloning of PCR product in to T vector

PCR yielded a specific amplicon of 780bp in HPV strain. The fragment was separated on 1% agarose gel and purified using gel extraction kit. The purified PCR product was quantified and then ligated with cloning vector using T4 DNA ligase enzyme. The ligated plasmid was transformed in to *E.coli* bacterial strain DH5α. The plates were screened for blue white colonies(fig 2).

Fig2: Blue white selection of the transformed bacterial cells in the Xgal-IPTG-Ampicillin-LB Agar.

Plasmid Isolation

Plasmids were isolated from the transformed cells by using alkaline lysis method. The isolated plasmid was then electrophoresed on 1% Agarose gel. The purified plasmids were subjected to double digested using *Bam*HI and *Eco*RI (Merck, India). After incubation at 37^{o}C for 2 hours the restricted product was electrophoresed on 1% Agarose gel. The release of the gene product was visualized on the gel.

Sequence data

The gene was identified by sequencing of plasmid. An approximately 781bp region of the E6 gene was sequenced at Eurofins, Bangalore. The sequence data was shown below. Nucleotide sequence analysis of gene was used to investigate the identity of E6 gene of HPV. To demonstrate the quality and accuracy of results provided from a public database, we compared sequences to their corresponding GenBank sequences. The sequence had "perfect" match (similarity, 99%) with sequences of their corresponding gene E6 from GenBank as determined by using BLAST (version 2.7).

Confirmation of clone by restriction digestion

The purified plasmid was subjected to restriction digestion using *Bam* H1 and *Eco*R 1 (Merck, India). After incubation at 37^{o}C for 4 hours the restricted product was electrophorized on 1% Agarose gel. The release of the gene product was visualized in the gel (Fig.3)

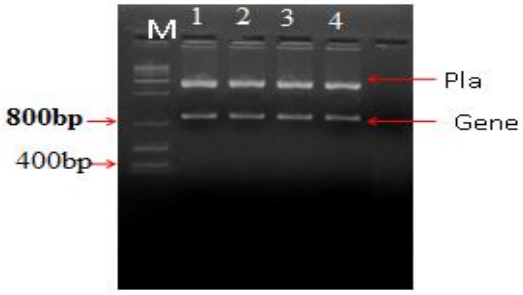

Fig.3: Restriction digestion of ligated plasmid using *Bam* H1 and *Eco*R 1 (Lane 1- restricted product, M-200bp ladder).

Sequence data: The gene was identified by sequencing of plasmid. An approximately ~800-bp region of the *E6* gene was sequenced at Eurofins, Bangalore. The sequence data was shown bellow**.** Nucleotide sequence analysis of gene was used to investigate the identity HPV E6 and *E7* gene. To demonstrate the quality and accuracy of results provided from a public database, the sequences were compared to their corresponding GenBank sequences. The sequence had "perfect" match (similarity, 99%) with sequences of their corresponding gene (E6&E7) from GenBank as determined by using BLAST (version 2.7). (Fig 5)

Cloning of *E6* gene into expression vector

The T vector clone was restricted with restriction enzyme (EcoR1 and *Bam*H1) and the released gene product was gel purified using gel extraction kit. The purified gene fragment was quantified and ligated with linearized pET20b expression vector (Fig 15) using T4 DNA ligase.

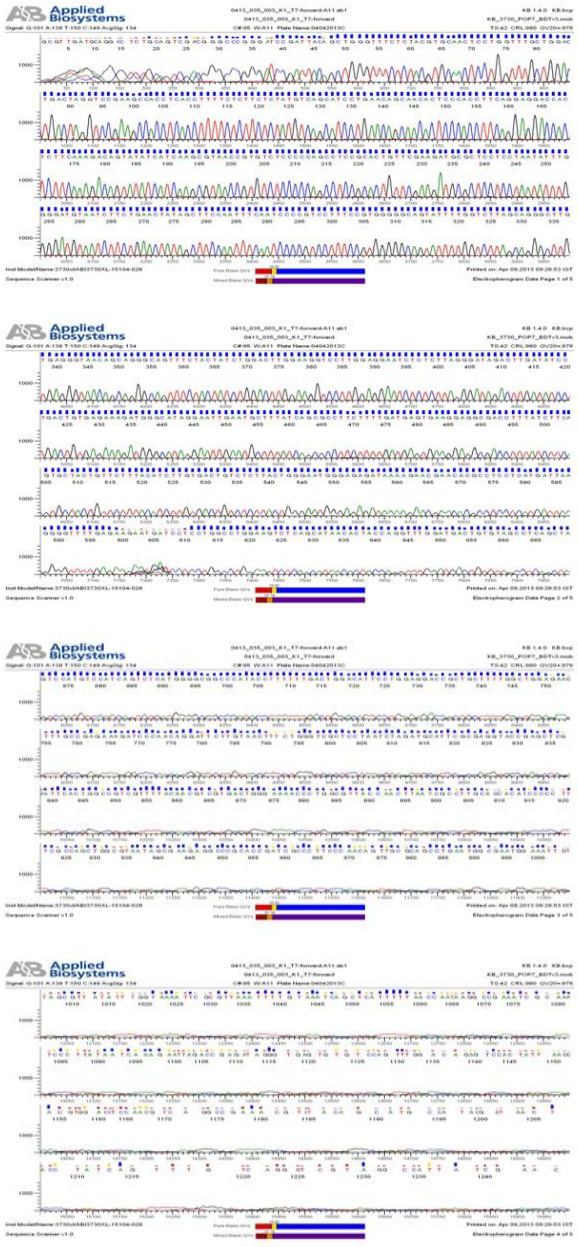

Fig5: Sequence data of E6 gene

SDS PAGE analysis of *E6* and *E7* gene expression

Inserted *E6* gene was expressed significantly in the prokaryotic expression system, and specific strip at ~ 18 kDa was demonstrated in SDS-PAGE (Fig 4). The E6 protein of HPV is an 18 kDa phosphoprotein, which is localized in the nucleus and in non-nuclear membranes. E6 is a critical factor in tumor formation and acts to destabilize the tumor suppressor p53. E7 is a 11 kDa protein with a zinc finger motif.

SUMMARY AND CONCLUSION

There are two major issues for the use of molecular HPV diagnosis. The first is to screen the women infected with cervical cancer, which can be done through community-based screening programs or through clinical counseling(Werness BA et al; 1990). The second

includes vaccination trials, epidemiological and natural history studies. In this case, contrarily to the clinical application, a highly sensitive and reproducible assay is required, and it should include the broadest spectrum of HPV types(Smith JS; 2004).

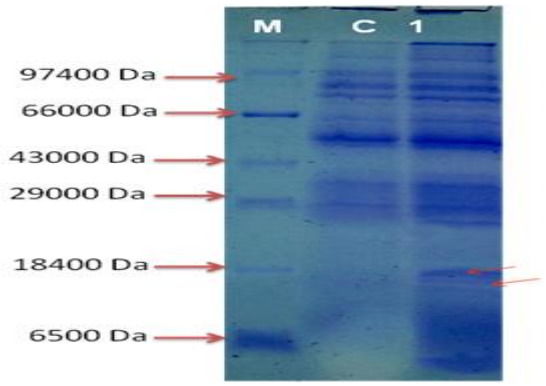

Fig.4: SDS PAGE analysis of *E6* gene expression (M- Protein Molecular weight ladder (Phosphorylase B-97400, Bovine Serum Albumin-66000, Ovalbumin-43000, Carbonic Anhydrase-29000, Lactoglobulin-18400, Aprotinin-6500) C-Control, 1-*E6 and E7* gene expression).

HPVs encode two major oncoproteins, E6 and E7, which are consistently expressed in cervical carcinomas. E6 and E7 lack intrinsic enzymatic activities and transform cells by stimulating cell growth and inactivating tumor suppressor pathways(Lacey JV et al; 1999). Expression of HPV16 E6 oncoprotein in primary human epithelial cells causes genomic instability(Koutsky L;1997).

In the present study the culture of HPV are amplified for the E6 gene. The primer was designed to amplify the oncoprotein gene E6. The purified product was cloned into T vector and identified using blue white screening. The positive clones were identified, digested and sequenced. The sequence was matching with E6 sequence of HPV 16 strains. The cloned oncoprotein gene was cut from the T vector and ligated with the expression vector pET20b and transformed in to *E.coli* strain for protein expression analysis. Proteins extracted were approximately 18kDa. A striking feature of all E6 proteins derived from the high risk HPV types is the presence of a highly conserved PDZ-binding domain. Though it is not involved in p53 binding and degradation, but contributes to E6 transforming activity. Hence transforming of the rodent cells with this gene might induce viral oncogenesis and the interactions might represent ideal therapeutic targets for the later stages of virus-induced disease.

REFERENCE

1. Anna-Barbara Moscicki, MD, Yifei Ma, MS and Stephen Shiboski, 2010; The role of sexual behavior and HPV persistence in predicting repeated infections with new HPV types, Cancer Epidemiol Biomarkers Prev. 19(8): 2055–2065.
2. Arends MJ, Wyllie AH, Bird CC. Human papillomavirus type 18 is associated with less apoptosis in fibroblast tumours than human papillomavirus type 16. Br J Cancer 1995;72:646–9.
3. Arens M. (2001) clinically relevant sequence-based genotyping of HBV, HCV, CMV, and HIV. J Clin Virol; 22:11–29.
4. Beasley RP, Trepo C, Stevens CE, et al. The E antigen and vertical transmission of hepatitis B surface antigen. Am J Epidemiol 1977;105:94–8.
5. Bosch FX, Lorincz A, Munoz N, Meijer CJLM, and Shah K V (2002).The causal relation between human papillomavirus and cervical cancer. *J Clin Pathol*. 55: 244 – 65.
6. Dillner J. (1999). The serological response to papillomaviruses. *Semin Cancer Biol*; 9:423–30.
7. Francheschi S, Rajkumar T, Vaccarella S, Gajalakshmi V, Sharmila A, Snijders PJF, Muñoz N,Meijer CJLM, and Herrero R, "Human papillomavirus and risk factors for cervical cancer

inChennai, India: A case-control study" *International Journal of Cancer*, 107 (2003): 127-33.

8. Gissmann L, Wolnik L, Ikenberg H, et al. Human papillomavirus types 6 and 11 DNA sequences in genital and laryngeal papillomas and in some cervical cancers.Proc Natl Acad Sci USA. 1983;80:560–3.
9. Hamid NA, Brown C, Gaston K. The regulation of cell proliferation by the papillomavirus early proteins. Cell Mol Life Sci. 2009 May;66(10):1700-17.
10. Hariri S, Unger ER, Sternberg M, et al. Prevalence of genital human papillomavirus among females in the United States, the National Health and Nutrition Examination Survey, 2003–2006.*Journal of Infectious Diseases* 2011; 204(4):566–573.
11. Jacobs MV, Walboomers JMM, Snijders PJF, et al. Distribution of 37mucosotropic HPV types in women with cytologically normal cervical smears: the age-related patterns for high-risk and low-risk types. Int JCancer 2000; 87:221–7.
12. Koutsky L. Epidemiology of genital human papillomavirus infection. Am J Med 1997;102:3– 8.
13. Lacey JV, Brinton LA, Abbas FM, et al. Oral contraceptives as risk factors for cervical adenocarcinomas and squamous cell carcinomas.Cancer Epidemiol Biomarkers Prev 1999;8:1079–85.
14. Lilienfeld AM. On the methodology of investigations of etiologic factors in chronic disease—some comments. J Chronic Dis 1959;10:41–6.
15. Munger K, Scheffner M, Huibregtse JM, et al. Interactions of HPV E6and E7 oncoproteins with tumour suppressor gene products. In: LevineAJ, ed. Tumour suppressor genes, the cell cycle and cancer. New York:Cold Spring Harbor Laboratory Press, 1992:197–217.
16. Neerja Bhatla & Nidhi Moda, Indian J Med Res 130, September 2009, pp 261-265, The clinical utility of HPV DNA testing in cervical cancer screening strategies.
17. Parkin DM. The global health burden of infection-associated cancers in the year 2002.*International Journal of Cancer* 2006; 118(12):3030–3044.
18. Sambrook, J. & Russell, D. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
19. Scheffner M, Munger K, Byrne JC, et al. The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. Proc NatlAcad Sci U S A 1991;88:5523–7.
20. Scheurer ME, Tortolero-Luna G, Adler-Storthz K. Human papillomavirus infection: biology, epidemiology, and prevention.*International Journal of Gynecological Cancer*. 2005;15(5):727–746.
21. Smith JS, Bosetti C, Munoz N, et al. Chlamydia trachomatis and invasive cervical cancer: a pooled analysis of the IARC multicentric case-control study. Int J Cancer 2004;111:431–9.
22. Tindle RW. Vaccines for human papillomavirus infection and and genital disease. Texas: RG Landes Company, 1999.209 de Villiers EM. Taxonomic classification of papillomavirus.Papillomavirus Report 2001;12:57–63.
23. Werness BA, Levine AJ, Howley PM. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science1990; 248:76–9.
24. Winer RL, Lee SK, Hughes JP, et al. Genital human papillomavirus infection: incidence and risk factors in a cohort of female university students. Am J Epidemiol 2003;157:218 – 26.
25. Zur Hausen H. Cervical carcinoma and human papillomavirus: on the road to preventing a major human cancer. J Natl Cancer Inst2001;93:252–3.
26. Zur Hausen H. Papillomavirus causing cancer: evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst 2000;92:690–98.