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Novel Method for Detection, Quantification and Cytopathic Changes in Developed Epithelial Cell Line with Human Papillomaviruses-16 Infection

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Authors' contributions

This work was carried out in collaboration among all authors. Authors RI and KPS were main worker. Authors PWR and MS refined the manuscript. Author LSS was clinically verify the sample and results. Author DKC designed, supervised and managed the study performed generated. All authors read and approved the final manuscript.

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ABSTRACT

Human papillomaviruses (HPVs) belong to the class of DNA viruses which are known to cause different human cancers. Development of new diagnostic methods and tools to study virus biology and mechanism of infection had been strongly felt in order to prevent HPV infections. In the present study we have designed new set of primers from E6 gene of HPV for its detection using PCR with an increased sensitivity of upto 0.2 pg. Hence, we established and characterized a new cervical epithelial cell line designated as CEC to be used as an *in vitro* tool for studying viral biology in order to prevent HPV infections. The interaction of HPV with CEC cells resulted in its neoplastic transformation and cytological changes such as multinucleation, nuclear irregularity, nuclear enlargement and perinuclear halos. CEC cell line was thus successfully established as an *in vitro* model system for studying interaction of HPV with squamous cervical epithelial cells.

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1. INTRODUCTION

Cancer of the cervix is the second most frequent gynaecological malignancy in the world [1]. In India the observed annual incidence rate is 130,000 with 70 -75000 deaths per year [2]. The uterine cervical epithelial cell protects the upper reproductive tract from insults providing the physical barrier, secretions containing bactericidal and virucidal agents and pathogen dependent direct immunomodulation [3-5]. Human papillomavirus is main causative factor for the infection [6-8] Certain type of viruses lead to cervical cancer and it can distinguish as high risk human papillomavirus (HRHPV) and low risk human papillomavirus (LRHPV) [9-11]. HRHPV like HPV16, HPV18, HPV31, HPV33 and HPV45 are associated with invasive carcinoma of cervix [12]. HPV comprises of the coding region for early genes (E1, E2, E4, E5, E6 and E7) which regulates the vegetative and productive phase of viral cycle [13]. E6 is known to block cell apoptosis by directing the p^{53} suppressor protein to the proteasome. Expression of the E6 protein suppresses the apoptotic response by the inappropriate activation of the E3 ubiquitin ligase E6AP, which ubiquitinates p^{53} labelling it for degradation by 26S proteasome [14,15]. Since the demonstration of the association between HPV and cervical cancer, many efforts have been made to develop HPV DNA detection methods. Over the past few years constant progress has been made on HPV typing based on polymerase chain reaction (PCR) methods. Multiplex PCR methods with high sensitivity and possibility to perform multiple amplification in a single reaction [16] had been used to detect DNA from HRHPV in 91% of the patient that were tested positive by the hc2 assay. HPV 16, 18, 31, 33 and 45 are the most common genotype reported to cause the infection.

Cervical cell cultures have served as *in vitro* model systems for basic research [17] and for oncological and microbiological studies [18-20]. It is important for clinicians to recognize histopathlogic and cytological lesions [21].

The present study was thus aimed at development and characterization of the cervical epithelial cell line (CEC) for studying cytopathic changes upon infection with HPV 16. A set of primers were also designed and synthesized from HPV E6 protein for sensitive detection of the viral infection.

2 MATERIALS AND METHODS

2.1 Sample Collection

Cervix brush samples from a 50 year old woman (with permission) were collected for detection of oncogenic human papillomavirus infection using the protocol for hybrid capture 2 (HC-2) test given by Karwalajtys et al. [22].

2.2 Genomic DNA Isolation

The isolation of genomic DNA from vaginal swab was done as per the method described by Hiney et al. [23] with some modifications. 500 µl lysis buffer (100 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 1.25% NaCl and 0.25%Sucrose) with 10µl proteinase K (10 mg/ ml) was mixed with 100 µl of swab sample and incubated at 37℃ for 2 hrs. Equal amount of phenol: chloroform: isoamyl-alcohol (25:24:1) was added to cell lysate and was mixed by inversion. The suspension was centrifuged at 10,000 rpm for 10 minutes at 40℃. The aqueous layer from the top was removed carefully to avoid protein debris and was transferred to a new tube. This step was repeated twice. 1/10 volume of sodium acetate (pH 7.0) and 2 volume of chilled ethanol was added to aqueous phase so as to precipitate the DNA and incubated at -20℃ for 30 minutes. The DNA was pelleted by centrifugation at 10,000 rpm for 20 minutes at -20℃. The pellet was washed with 1 ml of 70% ethanol and air dried and dissolved in 50 µl of TE buffer (pH 8.0) and stored at –20℃ for further study.

2.3 Designing of Specific Primers, PCR Conditions and Amplification

Human papillomavirus E6 gene sequence of type 16 was taken from NCBI (www.ncbi.nlm.nih.gov) and the nucleotide sequences were aligned in the ClustalW (http://align.genome.jp). Primers were designed from the highly conserved region using DNAstar software. The designed set of forward and reverse primer was synthesized commercially. The PCR reaction mixture (50 µl) consisted of 10 ng of genomic DNA, 2.5 units of Taq DNA polymerase, 5 µl of 10X PCR amplification buffer, 200 µM dNTP and 10 pmoles of each primer. Amplification included initial denaturation at 94℃ for 3 minutes, followed by 35 cycles of denaturation 94℃ for 1 minute, annealing of primers at 57.7℃ for 1 minute and primer extension at 72℃ for 1

minute. A final extension at 72℃ for 10 minutes was used. 10 µl of the reaction mixture was then analyzed by 1% agarose gel electrophoresis the reaction products were visualized under UV light.

2.4 Sensitivity of PCR and Cross-reactivity of Primers

The genomic DNA (20 pg) of HPV was diluted in 10 fold dilutions and amplification by PCR was done using the diluted DNA template with conditions as described above. The cross-reactivity of HPV gene primers were checked by NCBI Basic Local Alignment Search Tool (BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi) [24] and by genomic DNA amplification of other HPV type such as HPV type 18, 31, 33 and 45.

2.5 Purification and Sequencing

The amplified product was purified with gel elution kit (Qiagen) and sequenced. The nucleotide sequence of E6 of HPV16 was analyzed and deposited in NCBI GenBank.

2.6 Establishment and Characterization of Cell Line

2.6.1 Sampling

We collected the cervical tissue sample of HPV negative cervical epithelial cell of a noncancerous routine hysterectomy woman from King Georg medical university and immediately transferred it into the 10% FBS containing cell culture medium (L-15) at room temperature and transported it into cell culture laboratory.

2.6.2 Preparation of tissue for primary cell culture

The tissues were washed twice with phosphate buffer saline containing 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin, 1 µg/ml amphotericin B, 10 µg/ml reduced glutathione and 10 µg/ml ascorbic acid as antioxidants and transferred to a petri dish containing L-15 medium with 20% fetal bovine serum (FBS). The tissues were cut into small pieces with the help of sterile scissors and transferred to a 25 cm^2 flask and incubated overnight at 37℃. The tissues adhered to the surface of the flask. About 50% of the medium was replaced every 4^{th} day. After formation of monolayer, the cells were trypsinized with trypsin- EDTA solution (Invitrogen) and subcultured at a split ratio of 1:2 in L-15 medium. The concentration of FBS in medium was gradually reduced from 20 to 10% between 10^{th} and 20^{th} subculture. The cells were cryopreserved using a standard protocol *i.e* $5x10^4$ cells were resuspended in 500 µl cryomaxx and cooled down to -80℃ and were stored in liquid nitrogen after every 10^{th} passage.

2.6.3 Effect of temperature and FBS concentration on cell growth

To examine the effect of different temperatures on CEC cell growth, a total of $1x10^5$ cells were inoculated into 25 cm^2 cell culture flasks and incubated at 37℃ for 2 h for attachment of cells. Afterwards, the batches of culture flasks were incubated at selected temperatures of 28, 33, 37 and 42℃ for growth studies. The study was performed using L-15 medium supplemented with 20% FBS. Every day, three flasks at each temperature were trypsinized to measure cell density. The study was carried out for 5 days. Similar study was carried out to study the effect of different concentrations of FBS (5, 10, 15 and 20%) on cell growth at 37ºC.

2.6.4 Plating Efficiency (PE)

The plating efficiencies of CEC cell line were determined at passage 36^{th}. Tissue culture flasks (25 cm^2) were seeded with 100, 500, and 1,000 cells per flask and cultured in L-15 medium with 15% FBS at 28℃. Half of the medium was replaced every 2^{nd} day. After 10 days, the medium was discarded and cells were washed with PBS. Thereafter, the cells were fixed with methanol and stained with crystal violet. The individual colonies were counted under the microscope, and plating efficiency was calculated using the formula: PE (%) = (number of cell colonies/number of cells seeded) x100 [25].

2.6.5 Transfection

The CEC cell line at the 36^{th} passage was propagated in a 6-well plate at a density of $1x10^6$ cells per well. Following attachment the cells were washed and the sub-confluent monolayers were transfected with 2 µg of pEGFP eukaryotic expression vector (Clontech, USA) using lipofectamine 2000 (Invitrogen), and the green fluorescence signals were observed using Leica fluorescence microscope after 48h transfection [26].

2.6.6 Antibodies and immunofluorescence

Immunotyping of CEC cell line was carried out as per Mauger et al. [27]. Briefly, CEC cells were grown on sterile cover slips. The cells were subsequently fixed and permeabilized with methanol at −20℃ for 30 min. For immunostaining, the cover slips were pre-incubated with PBS containing 1% BSA for 1h at 37℃, and then incubated overnight at 4℃ with mouse anticytokeratin (pan), clone AE1/AE3 antibodies (Invitrogen) or mouse anti- vimentin antibodies (Invitrogen). In control cover slips, only PBS with 1% BSA was used in place of primary antibodies. After PBS washing, cells were incubated for 1h with rabbit anti- mouse IgG FITC conjugate (diluted 1:50 in PBS containing 1% BSA). The cover slips were washed again in PBS, mounted in VECTASHIED mounting medium (Vector Laboratories, Burlingame, CA) and observed under fluorescent microscope.

2.6.7 Chromosomal analysis

Standard procedure was followed for the preparation of the karyotype [25]. Briefly, the cells at 45th passage were incubated in a 25 cm^2 tissue culture flask until 70–80% confluency was attained at 37℃. Colchicine solution (Invitrogen) was added to the cells at a final concentration of 0.2 µg ml^{-1}. The cells were incubated for 2h at 37℃. After gentle pipetting, detached cells were collected by centrifugation at 200 g for 5 min at 4℃ and treated with a hypotonic solution of 0.56 % KCl for 20 min. Thereafter, the cells were fixed in acetic acid: methanol solution (1:3) for 5 min at room temperature. Slides were prepared using a conventional drop-splash technique and stained with 5% Giemsa solution. Chromosomes were observed and counted under a light microscope.

2.6.8 Cytopathic changes in cell line

1ml vaginal swab sample were taken from HPV infected women and centrifuged with 10,000 rpm at room temperature. The supernatant were diluted 10fold into PBS (cell culture grade) and passed through 0.22 µm syringe filter to remove other micro-organisms. The HPV purified sample was transferred into the cell culture flask of cervical epithelial cell line in triplicate and the cytological changes in the squamous epithelial cells were observed.

2.6.9 Neutral red uptake assay

Neutral red uptake assay was carried out to measure plasma membrane integrity in CEC cells following exposure to different concentrations of mercuric chloride, as per Repetto et al. [28]. Briefly, wells of a 96-well plate were seeded with 100 µl of 1×105 CSK cells ml−1 and incubated overnight at 28℃. Thereafter, the culture supernatant was removed and 100 µl of L-15 containing mercuric chloride (SRL, Mumbai, India) in increasing concentration (1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, and 500 µgml−1) was added to the wells in triplicates and incubated at 37℃ for 24 h. In control wells, 100 µl of growth medium was added. Following washing with PBS, 100 µl of neutral red working solution (40 µg ml−1 of growth medium) was added to the wells. The plate was again incubated for 2 h at 37℃ and then the neutral red solution was decanted. The wells were washed with 150 µl of PBS followed by fixation of cells by adding 5% glutaraldehyde for 2 min. Then, 150 µl of neutral red destaining solution was added to each well and the plate was kept on a plate shaker for 10 min. The optical density (OD) of the neutral red extract was taken at 540 nm in a microplate reader. Data were correlated by using graph pad prism 5.0.

3. RESULTS

3.1 DNA Based Detection of HPV 16 Infection

3.1.1 Designing of primers and detection using multiplex PCR

Genomic DNA isolated from the vaginal swab of three HPV suspected patients was taken as template for detection of HPV16 infection. A pair of specific primers targeting a 326 bp conserved region of the HPV E6 gene were designed and designated as HPV16e6F (5` TGTGTGTACTGCAAGCAACA 3`) and HPV16e6R (5` AGACATACATCGACCGGTCC 3`). Testing of 03 suspected samples using the designed primers resulted in confirmation of the HPV16 infection. Optimal concentration of $MgCl_2$ and primer annealing temperature for E6 gene amplification was determined to be 1.5 mM $MgCl_2$ and 57.5℃ (Fig. 1). The annealing temperature was determined through gradient PCR with positive control.

3.1.2 Sensitivity of PCR and cross-reactivity of primers

The amplification of E6 gene was observed when the concentration of template DNA ranged between 20 pg to 0.2 pg. Gene amplification

using PCR was observed between these concentrations of template genomic DNA. Bellow these concentrations, PCR product were not observed (Fig. 2).

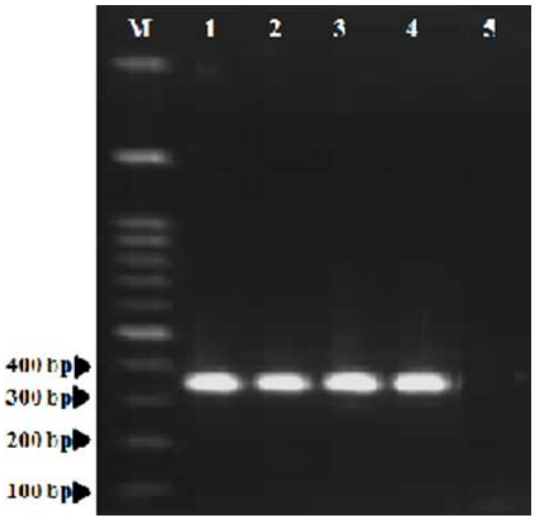

Fig. 1. PCR amplification of ~326 bp sequences of the HPV-16 genome using specific primers of the E6 genes, Lane M - 100 bp DNA ladder (Fermentas); Lane 1- Positive Control, Lane 2- Sample 2, Lane 3- Sample 3, Lane 4- Sample 4, Lane 5- Negative control

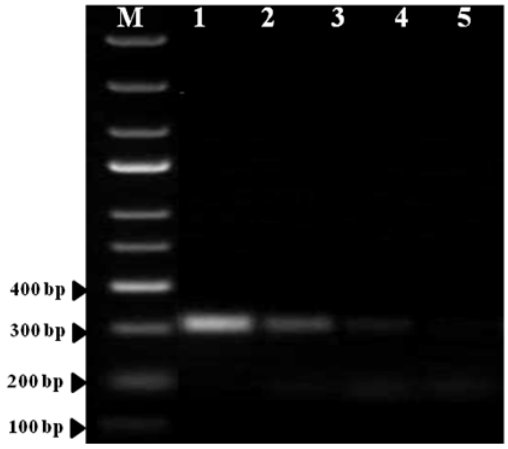

Fig. 2. PCR amplification and sensitivity of primer for E6 gene of HPV-16. Lane M: 100 bp Ladder; Lane 1: 20 pg; Lane 2: 2 pg; Lane 3: 0.2 pg; and Lane 4: 0.02 pg

3.1.3 Purification and sequencing

The amplified products of HPV16, E6 were purified using gel extraction kit (Qiagen) and the purified products were sequenced with our developed primers. Resulting sequences were analyzed and submitted to NCBI whose accession no is KR732977.

3.2 Establishment and Characterization of Cell Line

3.2.1 Development of primary culture from cervix tissue

We collected HPVnegative and noncancerous cervical tissue sample using routine hysterectomy of a woman from King George Medical University, Lucknow, India. Primary culture from cervical tissue explants was developed using standard explant method. Emergence of different types from the attached cervical tissue explants was observed within 5 days. However, simultaneous growth from all the explants was not observed. Small, epithelial-like cells were seen associated with many of the attached explants from the 3rd day onwards. The cells from primary explants showed both epithelioid and fibroblast-like morphology. However the epithelial cells continued to grow and formed a monolayer within 29 days. Initially, growth was very slow (Fig. 3).

3.2.2 Subculturing

Cells were sub-cultured in L-15 medium with 20% FBS at a ratio of 1:2 every 12-15 days for the initial 25 passages. After 25 passages, the cells were subculture at a ratio of 1:3 every 10 days. The concentration of FBS was gradually reduced from 20 to 10% from 15th to 25th passage. The cell line has been maintained for more than one year now and has been sub-cultured over 53 passages. This cell line has been designated as cervical epithelial cell line (CEC). The CEC cells were assessed for its revival efficiency after 5 months of storage in liquid nitrogen which revealed 70-80 % reviability and grew to confluency within 7 days. There was no alteration in morphology of cells during cryopreservation.

3.2.3 Growth studies of cells

CEC cells exhibited different growth rates at different incubation temperatures. The growth rate was moderate at 28, 33 and 42℃. Maximum growth of the cells was observed at 37℃ (Fig. 4A). The CEC cells grew moderately at 42℃ for first 3 days but thereafter; the cells showed vacuolation followed by rounding and

detachment. The growth rate also increased with increase in FBS concentration from 5 to 20% at 37℃ (Fig. 4B). The cells exhibited good growth at 10% FBS concentration though higher growth was observed at 15 and 20% FBS concentration.

3.2.4 Plating Efficiency (PE)

Plating efficiency of CEC cell line was determined at seeding concentrations of 100, 500 and 1000 cells per flask. The plating efficiency was found to be 4.97±1.57, 14.87±2.54 and 18.27±1.47, respectively. The efficiency improved with increase in seeding density.

3.2.5 Cell transfection

The cells at 39th passage were transfected with 2 µg of pEGFP vector. Strong green fluorescent signals were observed after 48 h of transfection (Fig. 5). The observed transfection efficiency was between 30–35% that indicated the expression of reporter gene GFP in CEC cell line.

3.2.6 Antibodies and immunofluorescence

All the CEC cells were strongly positive for cytokeratin, an epithelial cell marker (Fig. 6). No reactivity was observed in control cover slips and in cover slips incubated with anti- vimentin antibodies.

3.2.7 Chromosomal analysis

Chromosomal counts of 100 metaphase plates at passage 45 of CEC cell line revealed that the number of chromosomes in the cells varied from 24 to 56 (Fig. 7). The majority of the cells (68%) had a diploid chromosome number (2N=46) and the distribution was symmetrical.

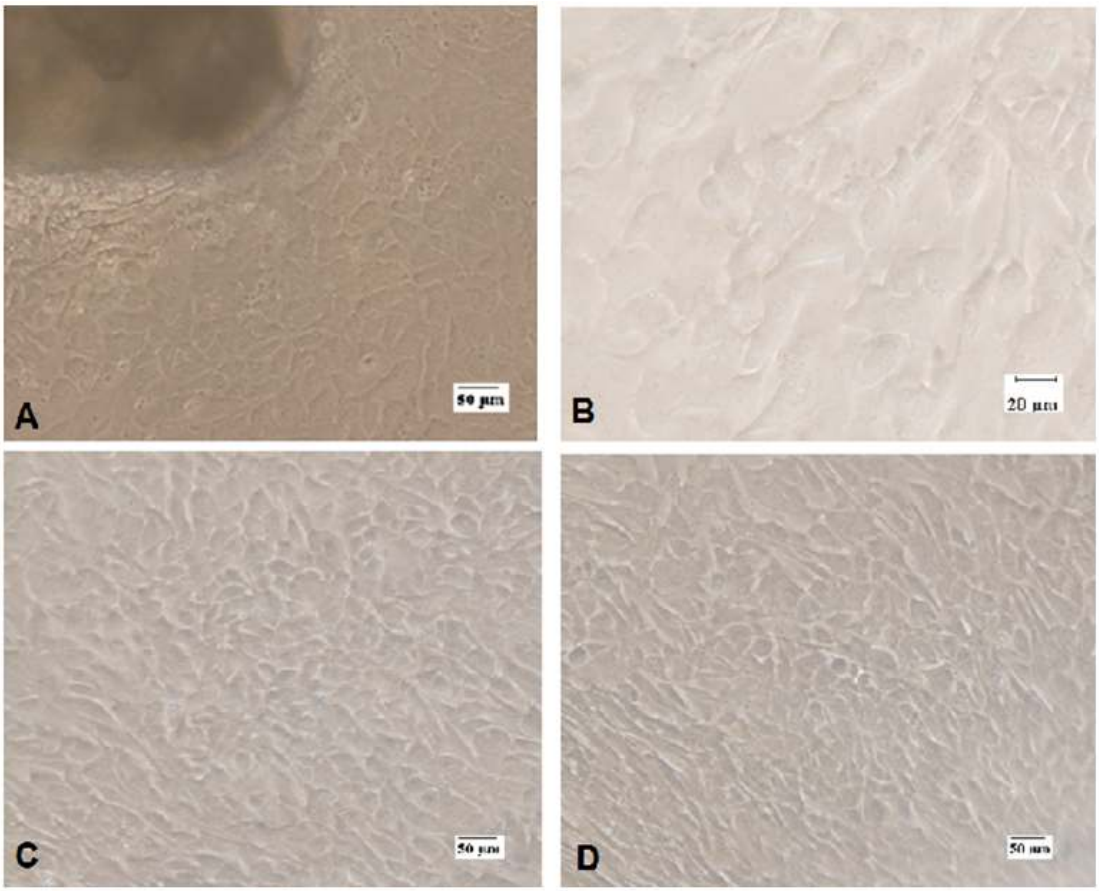

Fig. 3. Photomicrographs of CEC cells of Cervical epithelial; (A) Cervix explant showing radiation of cells; (B) Multiplying cells observed after 3rd passage; (C) Monolayer of CEC cells at 19th passage (D) Monolayer of CEC cells at passage 51st

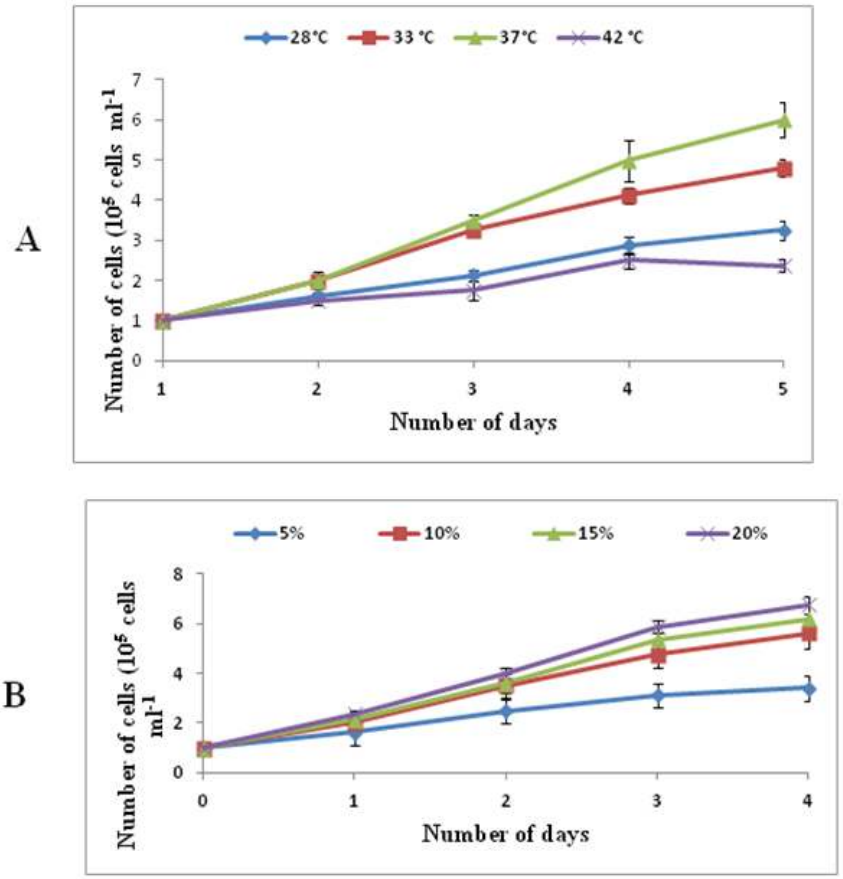

Fig. 4. Growth response of CEC cells at different temperatures at 32th passage (A); and different concentrations of serum at passage 33th (B)

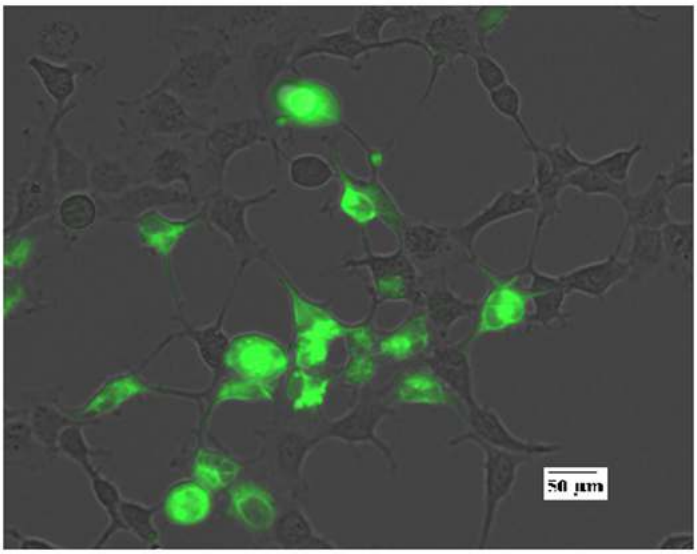

Fig. 5. The expression of GFP gene in CEC cells at passage 52 transfected with phrGFP II-N mammalian expression vector. (Scale bar= 200 µm)

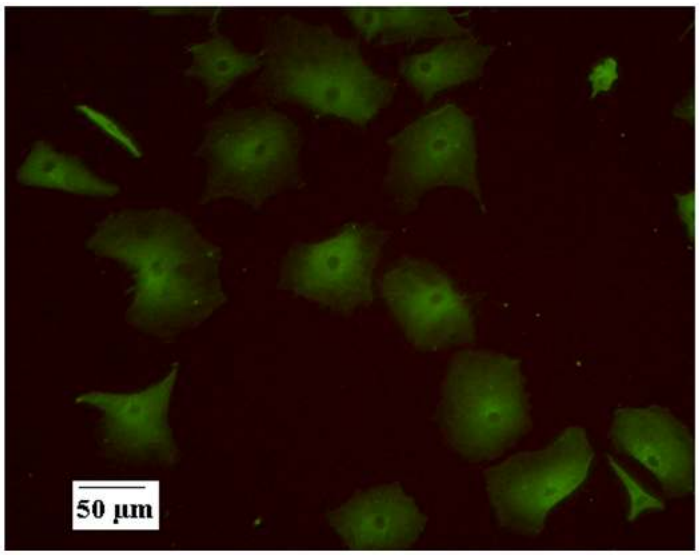

Fig. 6. Photomicrograph of CEC cells showing presence of cytokeratin

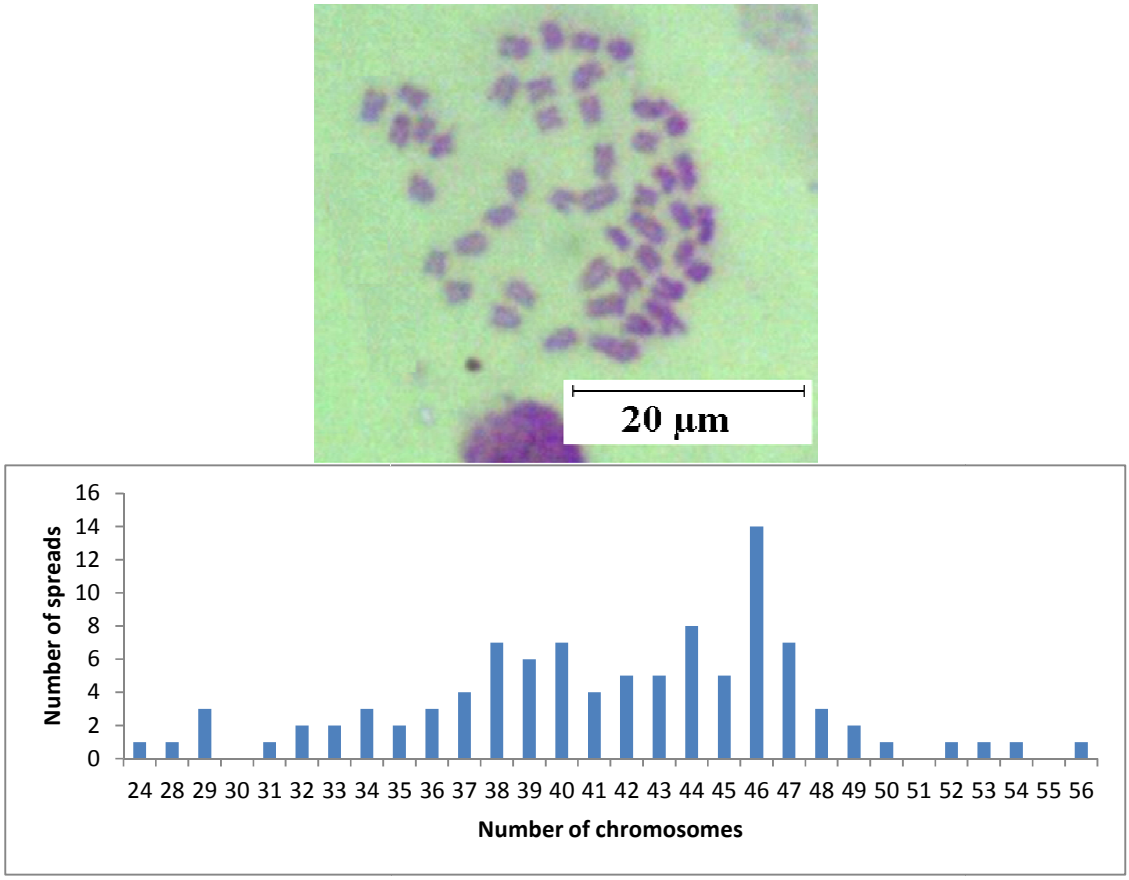

Fig. 7. Karyotype analysis of CEC cells at passage 35; (A) Chromosome number distribution, metaphase spread; and (B) Diploid karyotype of CEC cell. The main chromosome number was 46

3.2.8 Cytopathic changes in cell line

Inoculation of CEC cell culture flask using HPV purified sample obtained from HPV infected woman resulted in fast growth of the CEC cells and were ultimately transformed into neoplasia (Fig. 8). Cytological changes were observed in the squamous epithelial cells in comparison to the control flask (Fig. 9A). These changes observed were multinucleation, nuclear irregularity, nuclear enlargement and perinuclear halos (Fig. 9B)

3.2.9 Cytotoxicity assay

The CEC cells were incubated with L-15 medium containing increasing concentration of mercuric chloride and subsequently examined for effect on neutral red uptake. A decrease in O.D. was evident with increasing concentration of mercuric chloride (Fig. 10).

4. DISCUSSION

Cervical cancer is the most studied example for studying HPV infection which leads to neoplasia. High risk HPV infection is well known to interfere with the role and expression of cellular proteins that physiologically regulate cell proliferation and differentiation [29] leading to cervical cancer [30]. HPV has a double-stranded, circular DNA genome of approximately 7900 bp, with eight overlapping open reading frames and is known to causes a diverse range of epithelial lesions associated with the infection [31]. Cervical cancer progresses gradually from mild cervical intraepithelial neoplasia to severe neoplasia. Microinvasive lesions associated with the infection had been the basis for detection of HPV infection. Cervical cell suspension has been used routinely for DNA isolation and for detection of specific HPV DNA [32,33]. Serological tests or diagnostic methods based on antibodies

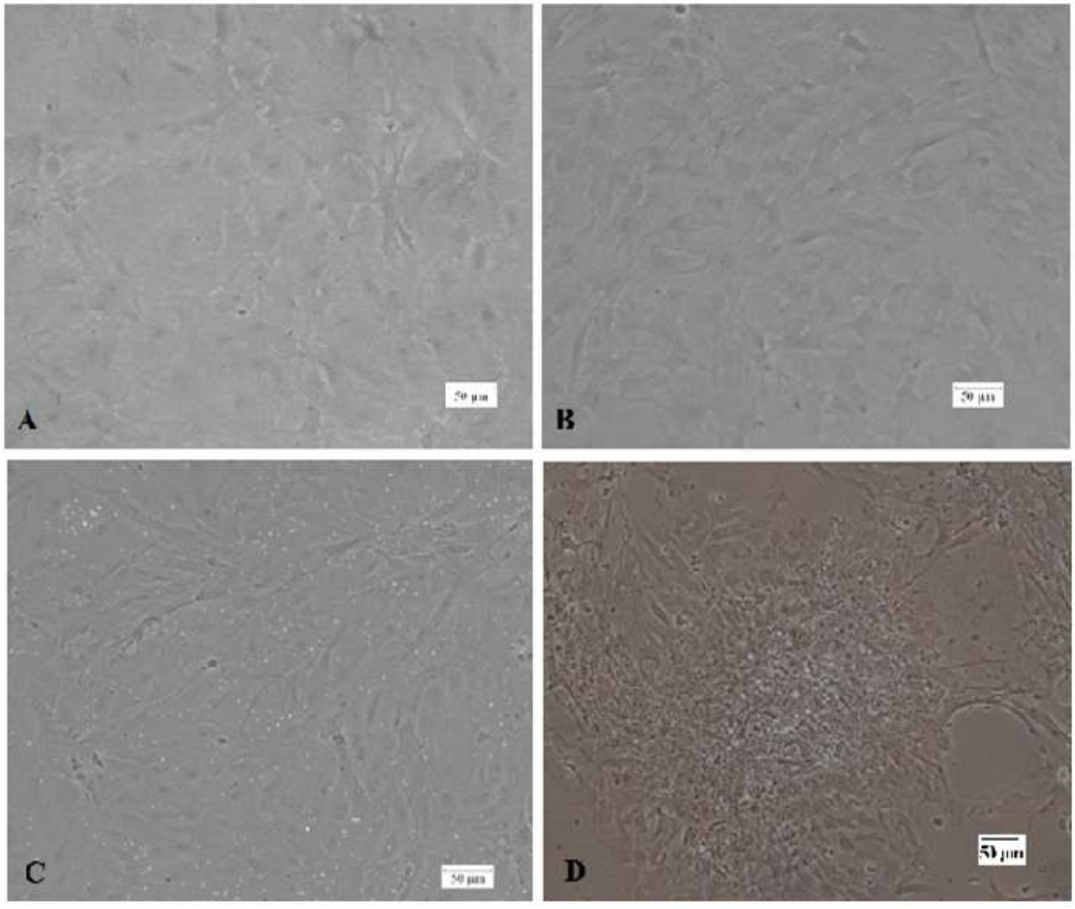

Fig. 8. Growth response of CEC cells With HPV infection. (a) A monolayer of CEC cells, (b) CEC cells at 24 h following inoculation of HPV, (c) CEC cells at 48 h post inoculation of HPV (d) CEC cells at 96 h post inoculation of HPV and showing malignant changes

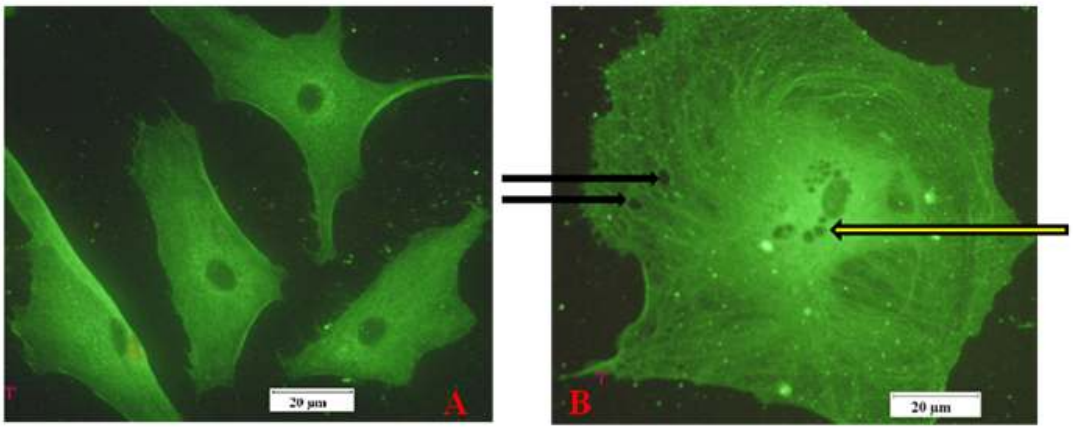

Fig. 9. Epithelial of cervix demonstrated the characteristic features of a productive HPV infection stained with cytokeratin. Fig. A is control and Fig B is showing multinucleation, perinulear halos, nuclear enlargement

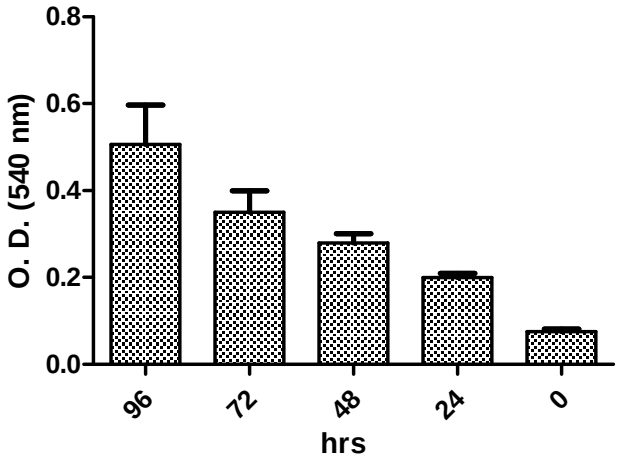

Fig. 10. Neutral red uptake assay; A. CEC cells were exposed to a range of concentrations of mercuric chloride (1.95 to 500 µM) for 24 h followed by incubation with neutral red medium for 2 h. A decrease in uptake of neutral red was evident with increasing concentration of mercuric oxide

had not been suitable for rapid diagnosis or distinguishing present and past infections [34]. Consequently, accurate diagnosis of HPV infection relies on the detection of viral nucleic acid [34]. Nucleic acid amplification methods have thus been developed to increase the sensitivity as well as the specificity of HPV-DNA detection [35]. The success of a PCR reaction ultimately depends upon the set of primers used for the reaction. In previous studies, we have found few reports on primers synthesized for sensitive detection of HPV16 E6 gene. Swan et al. [36] developed a primer of E6 gene with a detection sensitivity of 2.8 pg/ml, Duin et al. [37] designed a primer with sensitivity of 2 pg/ml, Weissenborn et al. [38] designed a primer with a sensitivity of 1.8 pg/ml, Lillo et al. [39] developed a primer with sensitivity as low as 0.8 pg/ml. In the present study, we have taken vaginal swab sample for DNA isolation and detection of HPV types through PCR. Here we developed a set of primers from E6 gene of HPV16 with an increased sensitivity of 0.2 pg/ml of DNA. As the HPVs are more vulnerable for the cervical cancer and currently there are no efficacious preventive measures or therapeutic tools to control the cervical cancer [40], the present set of primers will be helpful in detection of virus at an early stage.

Viruses can not grow up into non-living organisms and that's why the development of cell lines specific to virus infection becomes important to observe the cellular changes and vaccine development. Lee et al. [41] developed and characterized the cervical cell line by a non cancerous hysterectomy of cervix to understand the HPV genome replication and gene expression at an early stage of HPV genome establishment, and progression from initial infection to cancer. Sprague et al. [42] developed and characterized the cell line of cervix by using uterus as explants for studying viral replication. Miessen et al. [43] also established and characterized an epithelial cell line from porcine cervix to analyze the complex pathways within the cervical epithelium cells. In the present study we have established and characterized the cervical epithelial cell line from HPV negative cervical keratinocytes collected from a non-cancerous routine hysterectomy. Primary culture was established using explant method and the cell line had been sub-cultured for more than 53 passages. The cell line has been maintained in L-15 (Leibovitz) medium supplemented with 10 % fetal bovine serum. This cell line consists of homogeneous population of epithelial-like cells and grows optimally at 37℃. Karyotype analysis revealed that the modal chromosome number of CEC cells was 46 that confirmed CEC cell line originated from human. Significant green fluorescent signals were observed when the cell line was transfected with pEGFP mammalian expression vector, indicating its potential utility for transgenic and genetic manipulation studies. The CEC cells showed strong positive reaction for cytokeratin, indicating that the cell line was epithelial in nature. This cell line was susceptible to human papillomavirus, the causative agent of cervical cancer in women. Cytopathic changes in cervical epithelial cell line upon HPV infection revealed cellular enlargement, multi-nucleation, nuclear irregularity at different passages. Wright [44] had also observed similar cytological changes with low grade productive infection. He observed multi-nucleation, nuclear enlargement, nuclear hyperchromasia, and irregular nuclear outlines. Observed multi-nucleation is accounted for the genomic instability due to spindle pole defects [45] and due to the induction of multipolar mitosis [46]. This is the hallmark of cancer progression. French et al. [47] has reported epithelial carcinogenesis. Signaling of the transforming growth factor β (TGFβ) is known to control many cell processes, such as proliferation, differentiation, and survivability, and also involved in the pathogenesis of many different human diseases including cancer [48, 49].

5. CONCLUSION

In conclusion the developed primer will be useful for highly sensitive and specific detection of HPV16 infection from genomic DNA isolated from cervical swab sample. The established cervical epithelial cell line (CEC) will be of great help for isolation or propagation of HPV types; for development of an attenuated vaccine; for study of HPV biology and for development of *in vitro* immunoassay, inactivation assay and screening of HPV antiviral drug.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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