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 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. Usually E6 plays a crucial role in 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;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.

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. 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. 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 (Schneider 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 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 (Schneider 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, Francheschii 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 spectrophotometer (Vivacep 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’ AAACGTTCTGCTCATCTGC 3’ and the reverse primer, 5’-ATGTGCTGCCCAACACTTATT 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 MgCl₂ (1.5mM), 2μL of dNTP mix (2.5mM), 2μL 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 20μL. 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 visualized on a 1% agarose gel. The PCR product was recovered using the QIAquick 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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5’-3’)</th>
<th>GC %</th>
<th>Tm Value</th>
<th>Length (bp)</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>FW</td>
<td>AAACGTTCTGCTCATCTGC</td>
<td>54</td>
<td>60.4°C</td>
<td>20</td>
<td>781bp</td>
</tr>
<tr>
<td>TGC</td>
<td>0</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV</td>
<td>ATGTGCTGCCCAACACTTATTT</td>
<td>57</td>
<td>59.5°C</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>TTT</td>
<td>0</td>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cloning of the E6 gene

The purified PCR product was ligated into the pTZ57R/T cloning vector (Fermentas, USA) and the resulting plasmid was transferred 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 30 μl 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-5α 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 CaCl₂) 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 2 minutes. 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 agitum 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 120μg/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 transformant. 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 Tris-Cl, 1M 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 solution to the final was 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 up 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 12000 rpm for 5 minutes at 4°C. The supernatant collected was extracted with an equal volume of chloroform and isoamyl alcohol (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 BamHI (10U/μl) and EcoRI (10U/μl). The digested samples were resolved on 1% agarose gel and the released gene of insert was eluted from the agarose gel using gel extraction kit (Bolina 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 °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 dried, 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 electrophoresed 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 figure 1.

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).

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 BamH1 and EcoR1 (Merck, India). After incubation at 37°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 EcoR1 (Merck, India). After incubation at 37°C for 4 hours the restricted product was electrophoresed on 1% Agarose gel. The release of the gene product was visualized in the gel (Fig.3)

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 BamHI) 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).

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.


