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Functional characterization of E2 gene of high risk oncogenic human papilloma virus (HPV) 16

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ABSTRACT

In consistent to the fact that HPV sequence variations possibly influence virus carcinogenic potential, change in amino acid sequence of HPV-16 E5 protein might modify the transforming activity of the protein by affecting the interactions with the EGFR or potentially, other cellular proteins. In addition, it has been proposed that those variations in the E2 protein may affect the transforming potential of HPV-16 owing to changed affinity for cellular transcription factors or for viral DNA. In the present study, HPV 16 DNA was isolated from cervical cancer tissue using the specific primer designed by the Primer 3 plus software for the HPV antigen E2 gene. The amplified gene was ligated with T vector (pTZ57R/T) and transformed into DH5α cells. The plasmid DNA obtained was then confirmed by restriction digestion and sequence analysis. The sequence was found to be 99% similar to that obtained in GenBank. Dendrogram was constructed using ClustalW software to get the similarity of the sequence with the existing sequence in the NCBI. Further research is required to express the gene to get the protein antigen for the production antibodies or effective vaccine against high risk Papilloma virus.

Keywords: Human Papilloma Virus, E2 gene, Cervical cancer and PCR

INTRODUCTION

Globally, cervical cancer, one of the most common malignancies among women, is considered to be mainly caused by the oncogenic human Papilloma viruses (HPV 17, 18, etc) [1, 2]. Although more than 70 various types of human Papilloma virus genome have been identified, only few HPV types have been recognized as high risk types owing to their association with an genital cancers, particularly cervical cancer. It has been found that DNA of these high risk HPVs, chiefly HPV-16 and -18, are present in about 93% of invasive cervical cancer patients [3]. Despite the fact that the region of the Papilloma virus genome required for transformation was thought to be the long control region (LCR) and the E6 and E7 genes, E2 gene products also play vital role in either activating or repressing the transcription of the promoter. [4, 5, 6].

It has also been found that E6 and E7 promoters, which inactivate two important cellular tumor suppressors, the p53 and the pRB proteins, respectively, in HPV 16 is relatively repressed by E2. Although the role of HPV E2 proteins in transcriptional regulation has been studied extensively, sequence variation in E2 gene has not been studied comprehensively [7]. Moreover, the characterization of these high risk HPV types based on their gene sequence remains as a realistic method to identify different variants of HPV and thus it might demonstrate the alteration in normal physiological function [8]. As a result, the objective of the present study is to isolate HPV 16 DNA from the

cervical cancer tissue and then transform it into DH5α cells. The study also aims to verify the sequence of the E2 gene expressed by E.coli with reference to the GENBANK.

MATERIALS AND METHODS

Tissue sample collection

Cervical Cancer tissue samples were collected from Govt. hospital, Bangalore. A total of ten histology samples were collected and storied in -20°C.

Isolation of genomic DNA from cancer tissue

Total genomic DNA from the bacteria was isolated by N- Cetyl- N, N, N-trimethyl- ammonium bromide (CTAB) method. In order to isolate the genomic DNA, tissue was taken and homogenized in mortar pestle using liquid nitrogen. Followed by 675 µl of extraction buffer was added and incubated at 37°C for 30 min. Along with that, 75µl of SDS (20%) was added and incubated at 65°C for 2 hours. After incubation, samples were centrifuged at 10000 rpm for 10 min at 4°C. Clear solution was collected in a sterile micro centrifuge tube and added equal volumes of Chloroform: Isoamyl alcohol (24:1) and centrifuged at 10000 rpm for 10 min at 4°C. The aqueous phase was removed and taken in a sterile micro centrifuge tube and added 0.6 volumes of isopropyl alcohol and incubated at room temperature for 1hour followed by centrifuged at 10000 rpm for 10 min. Pellet was washed in 500µl of 70% ethanol using centrifugation at 10000 rpm for 10 min. Pellet was dried and dissolved in 20 µl TE buffer for 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.

Primer Designing and amplification of E2 gene

The specific primers were designed using Primer3 Plus software (http://www.bioinformatics.nl/ cgi-bin/primer3plus/primer3plus.cgi/) and the designed oligonucleotides were synthesized in Sigma Corporation USA. The details of primer for E used in our study are 2 gene amplifications are FP 5' GCC AAC ACT GGC TGT ATC AA3' and RP 5' GTC CTG TCC AAT GCC ATG TAG 3. The product size was 794pb. The PCR reaction conditions were optimized by changing the annealing temperature in the interval from 45 to 60°C and by altering the cycle number at which the phase change is initiated in the interval from 20 to 30 cycles. The highest amplification sensitivity and efficiency was obtained by an initial annealing temperature of 55°C for 30 cycles. The amplified product was electrophorized in 1% Agarose gel electrophoresis.

Cloning of the HPV16 (E2) genes and transformation

The eluted PCR products were ligated separately into the pTZ57R/T cloning vector (Fermentas, USA). A 30 µl ligation reaction was setup in 3:1 molar ratio of insert and vector DNA. 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. The ligated product was mixed with 200µl of prepared competent cells and incubated on ice for 30 minutes without disturbing. Heat shock was given to the ligation and competent cell mixture at 42°C for 2 minutes. The tubes were transferred quickly onto ice and incubated for 2-3minutes. To the mix, 1ml of LB broth was added and the tubes were placed 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 (50mg/ml) was added to a final concentration of 50 µg/ml, 200 µl of X- gal, to a final concentration of 80µg/ml and 20 µl of IPTG to a final concentration of 80 µg/ ml. The molten agar was mixed properly without forming air bubbles and poured on to the sterile Petri plates. The plates were allowed to solidify for 10-15 minutes and were incubated at 37°C until plating. After 1 h incubation in orbital shaker, the tubes containing cells were centrifuged at 1000rpm for 10 minutes at room temperature and resuspended the pellet in 100 µl of fresh LB broth. From the suspension, 100 µl was spread on LB agar plate using a bent sterile glass rod. The plates were incubated at 37°C overnight.

Screening of positive clones

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 transformants. All colonies from the master plate were subjected to plasmid DNA isolation and restriction analysis to identify the positive recombinants.

Confirmation of clones by restriction digestion
The purified plasmid was subjected to restriction digestion using restriction endonucleases (Bam H1 and EcoR1 (Merck, India) Restriction digestion was performed in 20 µl reaction volumes with recommended units of enzyme and appropriate buffers at 37°C for 4h. The products of restriction digestion were resolved in 1% agarose gel for confirming the release of the insert by the restriction endonucleases. The released gene insert was eluted from the Agarose gel using gel extraction kit (Bioline USA).

Phylogenetic analysis
Phylogenetic tree were generated on the basis of sequences of gene sequences using Clustal W 1.8 (Thompson *et al.,* 1994). The variable and incomplete sites at both the 5′ and 3′ ends of the gene sequences were excluded from the alignment. Sites presenting alignment gaps were excluded from analysis. A rooted phylogenetic tree was constructed using the sequences reported here with diverse sequences previously deposited in the GenBank database.

RESULTS

Genomic DNA isolation and quantification
The total genomic DNA was isolated by modified CTAB method. The quantity and quality of DNA was analyzed by UV visible spectrophotometer. The isolated DNA was then electrophorized in 1% Agarose gel as shown in the figure 1.

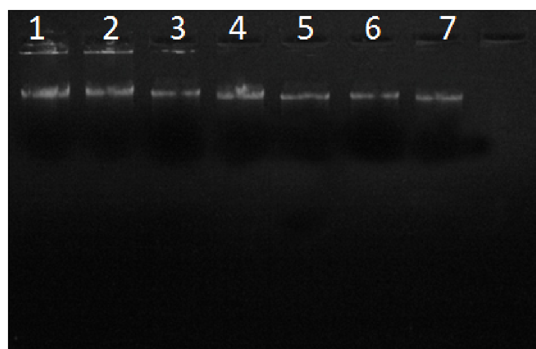

Fig. 1: Genomic DNA isolated from cervical cancer tissues (Lane 1-7 - Genomic DNA)

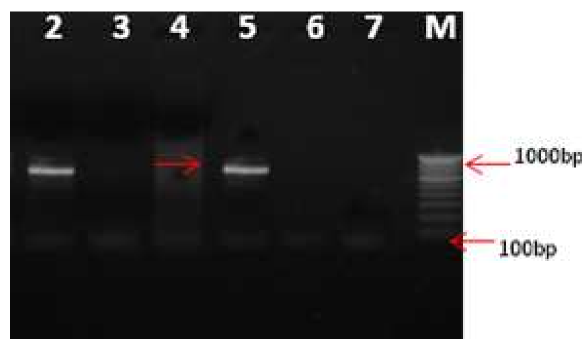

Fig. 2: PCR amplification of E2 gene by specific primers (M- 1kb ladder, Line 2, 5- E2 gene product, 3, 4, 6 & 7- Negative samples)

PCR amplification of the E2 gene
The strong association found between infection with HPV and the development of cervical neoplasia has been established in numerous case control studies. A prerequisite for studying the presence and variation of HPV in cervical lesions is a sensitive and high-resolution HPV detection system. The inherent sensitivity of PCR makes this method suitable for the analysis of even the most demanding clinical samples. Species specific primers were designed for the *Helicobacter pylori* using the sequences of E2 gene available in NCBI GenBank using Primer 3 Software. The predicted primers were validated initially *in silico* and subsequently in wet lab. The primers could

yield an amplicon of the expected size specific to E2 genes. The PCR product was electrophoresed and visualized by 1% agarose gel. The primers were found to produce ~800 bp amplicon.

Cloning of PCR product

As shown the previous studies, the viral oncogenes E2 is associated with cancer; authors are modified the cell cycle in order to favor the viral genome replication and consequent late gene expression. Most HPV-positive cancer cells maintain the expression of E2 (Shirasawa et al., 1987). A higher HPV16-E2 seropositivity was observed in patients with cervical cancer compared to healthy or with subjects displaying early lesions. Hence, E2 oncoprotein might be used to monitor infected woman at the very late stage of the infection. PCR yielded a specific amplicon of ~800-bp in three of the samples. The fragment was separated in gel and purified the product using gel extraction kit. The purified PCR product was quantified and ligated with cloning vector T vector pTZ57R/T (Fermentas, Germany) using T4 DNA ligase enzyme. The ligated plasmid was transformed in to *E.coli* bacterial strain DH5α. The transformation was done by heat shock method and transformed cell was cultured in the X gal-IPTG-Ampicillin-LB Agar plate at 37°C for overnight. The white colonies were picked up from the plates and cultured in Ampicillin containing LB broth as shown in the figure 3.

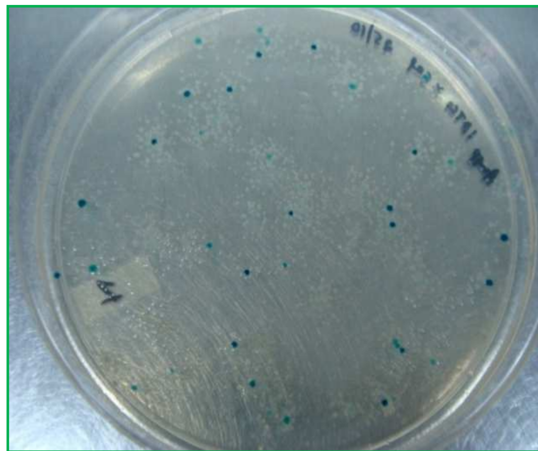

Fig. 3: Blue white selection of the transformed bacterial cells in the Xgal-IPTG-Ampicillin-LB Agar

Plasmid Isolation and Confirmation of clone by restriction digestion

Plasmid was isolated from the transformed cells by using alkaline lysis method. The isolated plasmid was electrophorized on 1% Agarose gel. The purified plasmid was subjected to restriction digestion using *Bam* H1 and *Eco*R 1 (Merck, India). After incubation at 37°C for 4 hours the restricted product was electrophorized on 1% Agarose gel. The release of the gene product was visualized in the gel as shown in the figure 4.

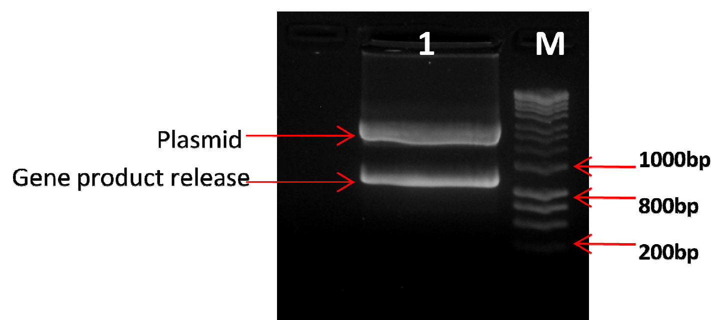

Fig.4: Restriction digestion of ligated plasmid using *Bam* H1 and *Eco*R 1 (Lane 1- restricted product, M- ladder)

Sequence alignment by clustalW

The gene was identified by sequencing of plasmid. An approximately ~800-bp region of the *E2,* gene was sequenced at SciGenome Kochi**.** Nucleotide sequence analysis of gene was used to investigate the identity HPV E2 gene. 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 (E2) from GenBank as determined by using BLAST (version 2.7). Cervical cancer detection worldwide and assess the geographic distribution of HPV genotypes, extensive epidemiological studies are required. Given the substantial genetic heterogeneity of HPVs and the possible clinical relevance of specific subtypes, specific molecular tools will be required. The E2 genes sequence of HPV is also available in GenBank database, which is more similar to this sequence (99% similarity). The N-J tree with branch length was plotted using ClustalW sequence alignment (http://align.genome.jp/), showing the relationship of *E6* and *E2* genes among the closest HPV in the NCBI database.

CLUSTAL 2.1 multiple sequence alignment of E2 gene

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AGAATAAAGCATTACAAGCAATTGAACTGCAACTAACGTTAGAAACAATATATAACTCAC 60
K02718-Human
AGAATAAAGCATTACAAGCAATTGAACTGCAACTAACGTTAGAAACAATATATAACTCAC 60
AF067031-Human
AGAATAAAGCATTACAAGCAATTGAACTGCAACTAACGTTAGAAACAATATATAACTCAC 60
AY686584-Human
AGAATAAAGCATTACAAGCAATTGAACTGCAACTAACGTTAGAAACAATATATAACTCAC 60
FJ610149-Human
AGAATAAAGCATTACAAGCAATTGAACTGCAACTAACGTTAGAAACAATATATAACTCAC 60
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HQ644297-Human
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E2
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AY686584-Human
CAATCCTCACTGCATTTAACAGCTCACACAAAGGACGGATTAACTGTAATAGTAACACTA 660
FJ610149-Human
CAATCCTCACTGCATTTAACAGCTCACACAAAGGACGGATTAACTGTAATAGTAACACTA 660
HQ644259-Human
CAATCCTCACTGCATTTAACAGCTCACACAAAGGACGGATTAACTGTAATAGTAACACTA 660
HQ644264-Human
CAATCCTCACTGCATTTAACAGCTCACACAAAGGACGGATTAACTGTAATAGTAACACTA 660
HQ644267-Human
CAATCCTCACTGCATTTAACAGCTCACACAAAGGACGGATTAACTGTAATAGTAACACTA 660
HQ644272-Human
CAATCCTCACTGCATTTAACAGCTCACACAAAGGACGGATTAACTGTAATAGTAACACTA 660
HQ644287-Human
CAATCCTCACTGCATTTAACAGCTCACACAAAGGACGGATTAACTGTAATAGTAACACTA 660
HQ644297-Human
CAATCCTCACTGCATTTAACAGCTCACACAAAGGACGGATTAACTGTAATAGTAACACTA 660
E2
CAATCCTCACTGCATTTAACAGCTCACACAAAGGACGGATTAACTGTAATAGTAACACTA 660

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AF067028-Human
CACCCATAGTACATTTAAAAGGTGATGCTAATACTTTAAAATGTTTAAGATATAGATTTA 720
K02718-Human
CACCCATAGTACATTTAAAAGGTGATGCTAATACTTTAAAATGTTTAAGATATAGATTTA 720
AF067031-Human
CACCCATAGTACATTTAAAAGGTGATGCTAATACTTTAAAATGTTTAAGATATAGATTTA 720
AY686584-Human
CACCCATAGTACATTTAAAAGGTGATGCTAATACTTTAAAATGTTTAAGATATAGATTTA 720
FJ610149-Human
CACCCATAGTACATTTAAAAGGTGATGCTAATACTTTAAAATGTTTAAGATATAGATTTA 720
HQ644259-Human
CACCCATAGTACATTTAAAAGGTGATGCTAATACTTTAAAATGTTTAAGATATAGATTTA 720
HQ644264-Human
CACCCATAGTACATTTAAAAGGTGATGCTAATACTTTAAAATGTTTAAGATATAGATTTA 720
HQ644267-Human
CACCCATAGTACATTTAAAAGGTGATGCTAATACTTTAAAATGTTTAAGATATAGATTTA 720
HQ644272-Human
CACCCATAGTACATTTAAAAGGTGATGCTAATACTTTAAAATGTTTAAGATATAGATTTA 720
HQ644287-Human
CACCCATAGTACATTTAAAAGGTGATGCTAATACTTTAAAATGTTTAAGATATAGATTTA 720
HQ644297-Human
CACCCATAGTACATTTAAAAGGTGATGCTAATACTTTAAAATGTTTAAGATATAGATTTA 720
E2
CACCCATAGTACATTTAAAAGGTGATGCTAATACTTTAAAATGTTTAAGATATAGATTTA 720

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AF067028-Human      AAAAGCATTGTACATTGTATACTGCAGTGTCGT 753
K02718-Human        AAAAGCATTGTACATTGTATACTGCAGTGTCGT 753
AF067031-Human      AAAAGCATTGTACATTGTATACTGCAGTGTCGT 753
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AY686584-Human      AAAAGCATTGTACATTGTATACTGCAGTGTCGT 753
FJ610149-Human      AAAAGCATTGTACATTGTATACTGCAGTGTCGT 753
HQ644259-Human      AAAAGCATTGTACATTGTATACTGCAGTGTCGT 753
HQ644264-Human      AAAAGCATTGTACATTGTATACTGCAGTGTCGT 753
HQ644267-Human      AAAAGCATTGTACATTGTATACTGCAGTGTCGT 753
HQ644272-Human      AAAAGCATTGTACATTGTATACTGCAGTGTCGT 753
HQ644287-Human      AAAAGCATTGTACATTGTATACTGCAGTGTCGT 753
HQ644297-Human      AAAAGCATTGTACATTGTATACTGCAGTGTCGT 753
E2                  AAAAGCATTGTACATTGTATACTGCAGTGACGT 753
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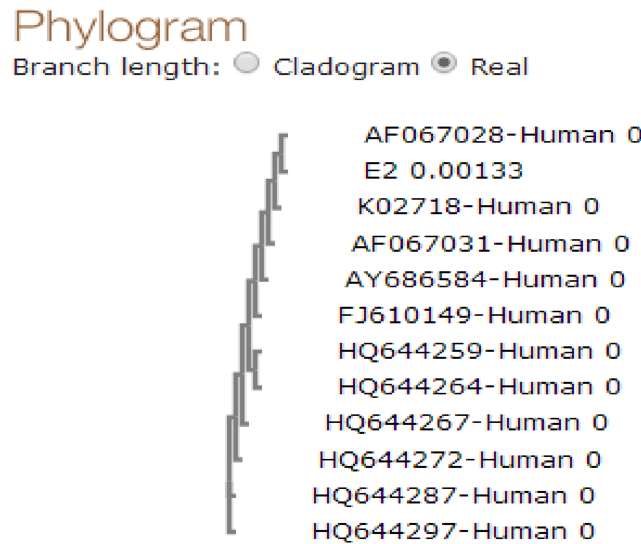

The sequence of HPV-E2 genes amplified by the specific primer is closely matching (99%) with a HPV strain but it is clustered differently Sequence analysis of the E2 genes from 12 strains was also carried out to obtain new insight into the genetic differences between the peptic ulcer group and the gastritis group. Interestingly, we found 4 mutations within the E2 open reading frame of our strains compared with previously published sequences in HPV. It is well known that local DNA sequences containing repeat sequences (direct repeats or inverted repeats) may cause deletion by misalignment during DNA replication or recombination.

DISCUSSION

So far, a variety of specific HPV variants have been considered to be related with the cervical neoplasia [9. 10]. It is well-recognized that the sequence variation in HPV E2 protein possibly affects HPV virus potential to be carcinogenic. Similarly, the study of HPV-16 E5 protein sequence has revealed that the change in amino acid sequence could modify transforming activity of the protein by influencing the interactions with the EGFR, the 16 kDa subunit of the H+-ATPase or, other cellular proteins [6, 11].

Moreover, a recent study by KA Jumaah et al 2014, which cloned and characterized the E6 oncogene of HPV 16 isolated from cervical cancer patients, has found that the E6 sequence was found to be 99% similar to the existing sequence in GenBank [12]. Consistently, the current study has also found that HPV antigen E2 gene isolated from cervical cancer tissue using the specific primer is 99% comparable to the sequence available in the database.

CONCLUTION

As the characterization of sequence variation within high-risk HPV types are potentially vital in the investigation of epidemiological association of various risk factors and cervical cancer, the current study harmonizes and widens HPV-16 genome sequence data as reported by previous studies [13, 14]. The present study, which cloned and characterized the E2 gene of HPV 16 virus isolated from cervical cancer tissues, demonstrated that the E2 sequence

is almost as comparable to the sequence in the NCBI database. The researchers also concluded that further studies are necessary to assess HPV-16 variant associations with cervical cancer risk and also, to characterize functional differences.

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