

Human papillomavirus in esophageal squamous cell carcinoma

Akshay Chandra Deka, Vidhya Srinivasan, Ananta Madhab Dutta¹, Pratap Chandra Sarma

Department of Pathology, College of Allied Health Sciences, ¹Department of Chemistry, Assam Down Town University, Panikhaiti, Guwahati, Assam, India

ABSTRACT

Context: We studied the role of Human papillomavirus (HPV) in esophageal squamous cell carcinoma from North East India. **Methods:** Fifteen archival paraffin embedded esophageal squamous cell carcinoma tissues were retrieved. Thin sections were made to extract DNA from the tissues. HeLa cell lines which harbor HPV DNA were used as a positive control. Nested polymerase chain reaction (PCR) was performed to analyze the presence of HPV DNA. Amplified products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. Positive product was sequenced for confirmation of the result. **Results:** HPV DNA was not able to detect in any esophageal squamous cell carcinoma tissue. Positive control HeLa cells DNA was found to be positive for HPV DNA by nested PCR. **Conclusions:** Our study shows the negative association of HPV in esophageal squamous cell carcinoma. However, further studies are needed with a large number of sample size to rule out the role of HPV in this region.

Key words: DNA, Human papillomavirus, esophageal squamous cell carcinoma, polymerase chain reaction

INTRODUCTION

Esophageal tumor is a tumor of esophagus. There are various subtypes, primarily squamous cell cancer and adenocarcinoma. Squamous cell cancer arises from the cells that line the upper part of the esophagus. Studies have shown that the incidence of esophageal tumor is higher in countries like India especially in North Eastern region. Esophageal carcinoma is a common malignancy, and its mortality rate is among the highest for cancers overall.^[1]

Etiology of esophageal cancer is not yet clear, but it is a multi-step progressive process. Major risk factors for esophageal cancer are not well-characterized, but may include poor nutritional status, low intake of fruits and vegetables, drinking beverages at high temperatures, tobacco usage and alcohol consumption.^[2-4] Tobacco and alcohol account for approximately 90% of all

esophageal squamous cell carcinomas. Tobacco smoking is also linked to esophageal adenocarcinoma though no scientific evidence has been found between alcohol and esophageal adenocarcinoma. Exogenous agents like coffee, tobacco, alcohol and nonvegetarian diet affect the methylation status of specific gene promoters in esophageal tissue and trigger epigenetic changes which may initiate the carcinogenic process.^[5,6]

It is reported from other part of the world the presence of Human papillomavirus (HPV) genome in esophageal tumor tissue and detected the presence of HPV 16 and 18. Most studies have failed to detect HPV DNA in esophageal tumors which were conducted in low-risk areas such as the USA or Europe.^[7-10] Studies in high-risk areas have found a significantly higher percentage of HPV in esophageal cancer.^[11-13]

The aim of the present study was to investigate the presence of HPV genome in esophageal squamous cell carcinoma in North East Indian population.

MATERIALS AND METHODS

Tissue samples

A total of 15 specimens of esophageal squamous cell carcinoma were used for this study. Archival paraffin blocks

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Address for correspondence: Mr. Akshay Chandra Deka, Department of Pathology, Assam Down Town University, Panikhaiti, Guwahati - 26, Assam, India. E-mail: akshay2k32001@yahoo.com

were obtained from Down Town Hospital Ltd., Guwahati, Assam. Four micro meter sections from each block were cut and stained with hematoxylin-eosin and examined for the confirmation of diagnosis. HPV positive cervical carcinoma HeLa cell lines that contain 10-50 copies of HPV-18 per cell were used as a positive control.

DNA EXTRACTION

On average 3-4 number of 4 µm sections were sliced from each paraffin block using disposable blades and gloves to avoid sample contaminations. DNA extraction was done by "QIamp® DNA mini kit" from Genetix Biotech Asia Pvt Ltd produced by QIAGEN company. (Which is most useful method of extraction method for paraffin embedded tissue sections). The extracted DNA was quantified at 260 nm wavelength of ultraviolet spectrophotometer. Adequacy and qualities of DNA was evaluated by polymerase chain reaction (PCR) amplification of human beta globin gene.

POLYMERASE CHAIN REACTION BETA GLOBIN

Extracted DNA was subjected to PCR for beta globin targeting 248 base pair fragment of the beta globin gene. PCO₄ (5'-GAAGAGCCAAGGACAGGTAC-3') and GH20 (5'-CAACTTCATCCACGTTCCACC-3') primer were used as forward and reverse primer respectively. The reaction mixture contained 10 mM of Taq buffer, 1.5 mM MgCl₂, 1.5 U of Taq polymerase, 200 µM each dNTP and 0.125 µM of each primer. PCR was done with the initial denaturation of 94°C for 4 min, followed by 35 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 45 s and a final extension at 72°C for 5 min. PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

NESTED POLYMERASE CHAIN REACTION FOR HUMAN PAPILOMAVIRUS

The degenerate MY09/MY11 primer set was used for the amplification of HPV DNA. The primer set (My09: 5'-GCACAGGGACATAACAATTGG-3' and My11: 5'-CGTCCAAAAGGAAACTG-3') was capable of amplifying a wide spectrum of HPV types to produce a PCR product of 450 bp. The amplification mixture consisted of 1x PCR buffer, (10 mM Tris/HCl, pH 8.3, 50 mM KCl, and 1.5 mM MgCl₂), 200 µM of each dNTP, 100 pmol of each primer, 2.5 units of Taq DNA polymerase (Bangalore Genei, cat-105926/Old No.MME5M and 500 ng of DNA in a final volume of 25 µl. Thirty-five cycles were completed as follows: 45 s at 94°C, 45 s at 45°C, and 45 s at 72°C. The initial denaturation step was for 3 min at 94°C, and a final

extension step was prolonged to 5 min at 72°C. Each batch of samples included negative controls containing water and positive control DNA from an HPV positive HeLa cell lines. The first round amplified product was used in the second round PCR. Thirty-five amplification cycles were completed according to the following protocol: Initial denaturation was for 5 min at 94°C, followed by denaturation for 1 min at 94°C, annealing for 2 min at 45°C, extension for 1.5 min at 72°C, and a final extension step of 5 min at 72°C. Each batch of samples included first round negative control and first round positive control product. GP05/GP06 primer set, a nondegenerate primer (GP05: 5'-TTTGTTACTGTGGTAGATAC-3' and GP06: 5'-GAAAAATAAACTGTAAATCA-3') was used for second round PCR, which detects a PCR product of approximately 150 bp. PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

DNA SEQUENCING

DNA sequencing was done for the amplified product of 2 µl round PCR. The amplified DNA product subjected to agarose gel electrophoresis to visualize the bands. The specific band for HeLa positive sample eluted from the gel and purified using QIAquick gel extraction kit produced by QIAGEN company as Genetix Biotech Asia Pvt Ltd. DNA ranging from 70 bp to 10 kb can be purified). The eluted Products were sequenced, and the sequence results verified using the National Center for Biotechnology Information nucleotide sequence viewer (<http://www.ncbi.nlm.nih.gov/BLAST>).

RESULTS

A total of 15 esophageal squamous cell carcinoma tissues were retrieved from Down Town Hospital of Assam, North East India. All the 15 specimens were from male having age range 39 to 86 years and a mean age of 58.06 years. All the tumors were well differentiated. Beta globin PCR was done to check the quality of the DNA. We performed nested PCR for serially diluted HeLa cell DNA to determine the sensitivity of the nested PCR which detects 145 base pair of the HPV DNA. Till 10⁻² dilutions, the nested PCR could amplify HeLa cell DNA [Figure 1]. HPV DNA was not able to detect in 15 esophageal squamous cell carcinoma. Positive control HeLa cells showed band specific for HPV DNA by nested PCR. The primer sets used detects the HPV consensus L1 region. The amplified product was runned in 2% agarose gel electrophoresis. None of the tissue specimens showed band for HPV genome whereas HeLa cell DNA showed band specific for HPV DNA [Figure 2]. The presence of HPV genome in HeLa cells were confirmed by DNA sequencing.

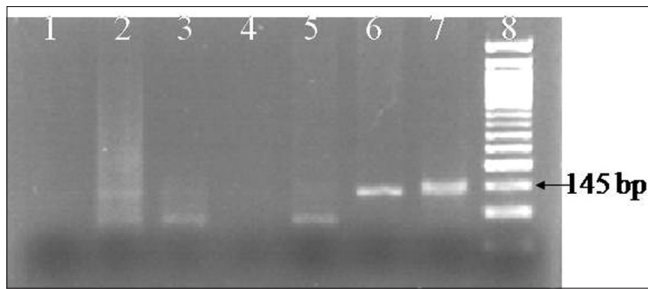


Figure 1: The sensitivity of the polymerase chain reaction for positive control DNA. Lane 1-7: Serial dilution of human papillomavirus DNA from 10⁻⁷ to 10⁻¹

DISCUSSION

This investigation was a retrospective study to determine the presence of HPV in esophageal squamous cell carcinoma in North East India. We were unable to detect HPV DNA in esophageal squamous cell carcinoma in this study. The PCR amplified DNA product for positive control were sequenced for the confirmation of HPV DNA. The sequence result shows the presence of HPV genome in the positive HeLa cells, confirming our sensitivity and specificity of PCR.

A limitation of this study, common to all studies that use L1 amplification, is that in the minority of cases HPV integrates to host genome and parts of L1 might be deleted. Therefore, although HPV is present, it is not amplified, and the test becomes false negative.^[14] A number of these cases are small, and it is unlikely that it highly affected the results of the current study.^[15] Moreover, employment of formalin fixed paraffin embedded tissue is another limitation of this study.

Most of the studies that suggested a role for HPV in esophageal systemic sclerosis were based on PCR. Being extremely sensitive method, conventional-PCR, and especially nested-PCR are prone to contamination by postPCR products that cause false positive results.

Mohiuddin *et al.* explained that the possibility that HPV in the esophagus may be behaving like an opportunistic commensal/pathogen, thereby the higher prevalence in normal esophagus. It is possible that some subtypes (so-called low-risk) of HPV may be living in synergy with the esophageal microenvironment, however in the presence of certain factors like exposure to harmful diet, tobacco, alcohol, free radicals, weakened immune system etc., they may be replaced by the pathogenic forms.^[16]

Dolgin has reported the presence of commensal viruses by sequencing based methods. It may be worth examining this kind of possibility by looking at cases with multiple infections, what sub-types they are, the individual histopathology and follow-up on response and survival.^[17]

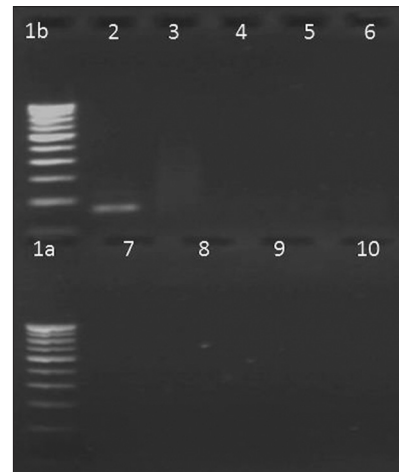


Figure 2: Polymerase chain reaction for tissue sample DNA and HeLa cell line DNA. Lane 1a and 1b: DNA ladder. Lane 2: Positive control band. Lane 3-10: PCR product for tissue sample DNA.

The association between HPV and esophageal squamous cell carcinoma is variable in different geographical areas, big sample size and also depends on the methods of detection used. Further studies are needed to understand the oncogenic roles of HPV in this region.

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