

The Association of Beta-catenin Gene Mutations and Human Papillomavirus in Carcinoma of Esophagus in a High-Risk Population of India.

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Abstract:

Background: Esophageal cancer (EC) is the sixth leading cause of death from cancer. In high-risk regions, squamous cell carcinoma is the most common type of EC, and its etiology remains poorly understood. It shows uneven geographical distribution in its occurrence, reflecting the influence of local environmental conditions, lifestyle and genetic predisposition in the development of the cancer. Kashmir, in the north of India, has been described as a high-risk area for esophageal squamous cell carcinoma (ESCC). In the present investigation an attempt was made to study the role of β -catenin mutations and human papillomavirus in 62 ESCC patients from Kashmir.

Methods: The hot spot mutation region of β -catenin exon 3 was evaluated in matched tumor and normal tissues using a combination of PCR-SSCP and direct sequencing. We used two different sets of consensus primers viz., GP5+ and GP6+; PGM09 and PGM11 in conjunction with reverse line blot assay to screen for human papillomavirus(HPV).

Results: None of the tumors showed the presence of commonly reported mutations in β -catenin. In view of the fact that HPV has been linked to pathogenesis of EC, we screened all the tumor and control specimens for the presence of HPV and we didn't detect HPV in any of the matched tumor and control specimens in contrast to the positive controls we used.

Conclusion: In conclusion our results suggest that squamous cell carcinoma of esophagus in Kashmir may arise independent of oncogenic β -catenin mutations and HPV is unlikely to be an etiologic factor for ESCC in this region.

Key words: Esophageal cancer, β -catenin gene mutations, human papillomavirus

Abbreviations: HPV-human papillomavirus, EC-esophageal cancer, ESCC-esophageal squamous cell carcinoma, PCR-polymerase chain reaction, Rb-retinoblastoma

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Introduction

Esophageal cancer is the sixth most common cause of cancer deaths worldwide and shows wide intra and international variation in incidence with ~ 462,000 new cases and 386,000 deaths each year.^(1,2) Over 80% of EC occur in developing countries, and most are the squamous cell type.^(1,2) One of the most intriguing features of ESCC is the wide variation in the disease incidence in different geographical regions of the world.^(1,3) In most countries, the incidence rates are around 2.5 to 5.0 per 100,000 for men and 1.5 to 2.5 for women. However, in distinct areas the incidence rates are remarkably higher, varying up to 500 fold from one area to another.⁽⁴⁾ A high incidence Asian “esophageal cancer belt” stretching from Northern Iran through the central Asian republics to North-central China, has been identified in the world.⁽⁵⁾ Kashmir borders the esophageal cancer belt on the south with incidence of 27 and 42 per 100,000 individuals in females and males, respectively, the highest incidence in India.⁽⁶⁾ The reasons for these major regional variations in the incidence of this disease are poorly understood. In western countries, where the risk of ESCC is generally low, consumption of tobacco and alcohol could explain more than 90% of the cases of ESCC.⁽⁷⁾ However, in countries with the highest rates of ESCC, such as Iran, China and India, only small proportion of ESCC could be attributed to smoking or alcohol consumption.^(4,8,9) Also, in high-risk populations, low consumption of fruits and vegetables^(4,10), nutritional deficiencies⁽¹¹⁻¹³⁾, poor oral hygiene⁽¹⁴⁾, nitrosamine exposure,^(15,16) and drinking hot liquids^(9,10,17,18) are considered to be risk factors for ESCC. The high incidence of ESCC has been linked to unique dietary habits which expose the population to a range of nitroso and other compounds.^(9,15)

The human Papillomavirus has been thought of as a possible etiological factor of EC since 1982 and there is a large amount of variation in the observations of such studies which have been reviewed by Syrjanen.⁽¹⁹⁾ The two transforming proteins E6 and E7 of high-risk HPVs can functionally interfere with the cell cycle control by interacting with tumor suppressor proteins p53 and Rb and it leads to loss of growth suppressive functions of p53 and Rb genes resulting in abnormal cell proliferation and growth of tumor.

Beta-catenin is a multifunctional protein that promotes cell proliferation by induction of gene transcription through activation of transcription factors like T cell-factor (TCF) and lymphoid enhancer factor (LEF).⁽²⁰⁾ In the absence of growth or differentiation signals, cytoplasmic beta-catenin is kept low, however mutations in the serine/threonine sites in exon 3 of β -catenin gene result in stabilization of β -catenin.⁽²¹⁾ Stabilization of β -catenin followed by nuclear translocation and subsequent TCF/LEF mediated transcriptional activation of specific genes, such as cyclin D1 and *c-myc* has been proposed as an important step in oncogenesis.⁽²²⁾ β -catenin mutations have been reported in both cell lines and human tumors.^(21,23) About 50% of the colon cancers with wild type APC (adenomatous polyposis coli) gene showed mutations in β -catenin⁽²³⁾ and such mutations have also been detected at a lower prevalence, in a variety of other cancers including medulloblastoma⁽²⁴⁾, uterine endometrial carcinoma⁽²⁵⁾, hepatocellular carcinoma⁽²⁶⁾ and prostatic adenocarcinoma.⁽²⁷⁾

In the present study, we report the screening for genetic alterations in the β -catenin gene involving exon 3 by single-strand conformational polymorphism (SSCP) followed by DNA sequencing and infection of HPV in 62 ESCC tumor and matched normal tissue samples of the patients from Kashmir, India.

Methods

Selection of Patients and Tissue Samples

This study was approved by the Ethical Committee of the Sher-i-Kashmir Institute of Medical Sciences, Srinagar, India. Informed consent was obtained from all the subjects included in the study. Pairs of ESCC tissue and corresponding normal mucosa were obtained from 62 Kashmiri patients who underwent surgery in the Department of Cardiovascular Thoracic surgery between 2001 and 2005. No patient had received chemo- or radiotherapy before surgery. In all cases, the histopathological type of tumor was squamous cell carcinoma. Cancer tissues and well-separated normal esophageal tissue obtained from surgically resected specimens from ESCC patients were immediately snap-frozen, and these were kept in liquid nitrogen. Genomic DNA was prepared by proteinase K digestion and phenol/chloroform extraction, followed by ethanol precipitation as described earlier.^(9,28)

Screening and Typing of HPV

Purified genomic DNA was amplified by PCR using two sets of primers (GP5+; GP6+ and PGMY09; PGMY11) with primers of β -globin gene as internal control (Table 1). Each batch of samples included negative controls containing water and positive control DNA from HPV positive cervical carcinoma and HeLa cell line. PCR products were analyzed on 8% polyacrylamide gel (Acrylamide: Bis-acrylamide, 29:1) and visualized by ethidium bromide staining. HPV genotyping was performed on all ESCC samples including positive controls. For PCR, 5 μ l of the DNA sample was amplified using a cocktail of biotinylated PGMY 09/11 and β -globin primers in final volume of 100 μ l. The PCR product was denatured in 0.4 NaOH, and subjected to HPV genotyping by the prototype PCR-line blot assay as described earlier.⁽²⁹⁾

The matched samples (tumor tissue and normal squamous epithelium of the same patients), were screened for mutations in the exon 3 of the β -catenin gene by PCR-SSCP (single-strand conformational polymorphism) and direct sequencing. The forward and reverse primer sequences for exon 3 of β -Catenin gene are given in Table 1. SSCP was performed using loading solution (95% formamide, 20mM NaOH, 0.025% bromophenol blue, and 0.025% xylene cyanol). 2 μ l PCR amplified product was added to 8 μ l of loading solution and

denaturation was done at 95 °C for 7 minutes and the sample was instantly cooled on ice for 5 minutes. Samples were loaded onto a 12% polyacrylamide gel (Acrylamide : Bis-acrylamide, 29:1, 10% glycerol, 0.5X TBE buffer) and run for 20 h in a SE 600 vertical gel apparatus (Hoefer Scientific, San Francisco, CA) at 150V. Gel temperature was regulated at 14 °C with a cooling circulating bath. After electrophoresis the gel was stained with silver nitrate to visualize DNA bands.

DNA Sequencing

Fresh PCR products were prepared by PCR amplification of original tumor DNA and its normal counterpart and purified by Qiagen PCR purification columns (Qiagen GmbH, Hilden, Germany). Purified products were used for direct DNA sequencing employing an automated DNA sequencer. To minimize the sequencing artifacts induced by PCR, products from at least 2 different PCRs were sequenced using forward and reverse primers with Big Dye terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems) based on fluorescence-labeled dideoxy nucleotides as chain terminators. Extension products were purified by ethanol precipitation as described by the manufacturer. Reactions were resolved on a model 3100 Avant Genetic Analyzer (applied Biosystems).

Table (1). Primer sequences used for the PCR amplification in the present study Mutational analysis: exon 3 of the β -catenin gene.

Target	Primer sequence (5'-3')	Approximate size (bp)
β -Catenin Exon 3	Forward	GCTGATTTGATGGAGTTGGA
	Reverse	GCTACTTGTTCTTGAGTGAA
HPV L1 gene	GP5+	TTTGTTACTGTGGTAGATACTAC
	GP6+	GAAAAATAAACTGTAAATCATATT
	PGMY09	CGTCC(C/A)A(G/A)(G/A)GGA(T/A)ACTGATC
	PGMY11	GC(C/A)CAGGG(T/A)CATAA(T/C)AATGG
β globin	Forward	GAAGAGCCAAGGACAGGTAC
	Reverse	CAACTTCATCCACGTTACACC

Results

All the matched 62 ESCC samples and adjacent normal tissues were screened for mutations in exon 3 of the β -catenin gene. None of the samples exhibited mobility shifts by SSCP analysis. We used a colon cancer sample as a positive control harbouring TCT-TTT (Ser⁽³⁷⁾-Phe) mutation, which consistently showed aberrant bands by PCR-SSCP method (Fig. 1) employed in the present study. In order to rule out the possibility that some mutations might remain undetected by SSCP, we performed the direct sequencing of exon 3 of β -catenin gene in at least 20 random tumor tissues and four control samples which also did not reveal any mutation.

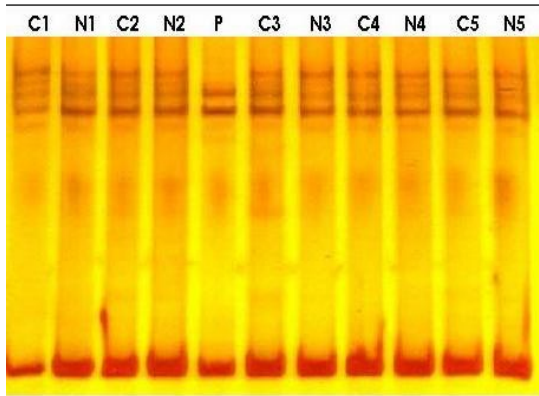


Fig. (1). PCR-SSCP analysis of β -Catenin exon-3 in esophageal tumor samples. Lanes representing C1, N1; C2, N2 etc. are matched DNA samples isolated from tumor tissue and normal surrounding tissue respectively. P is the positive control for β -catenin mutation (DNA from colon cancer specimen with a known TCT-TTT (Ser³⁷-Phe) mutation). As indicated there was no mutation found in exon-3 of all 47 matched squamous cell carcinoma samples from Kashmir

For amplification of HPV DNA, PCR using Gp5+/Gp6+ and PGMY09/PGMY011 primer pairs resulted in amplification of the appropriately sized DNA fragments in positive controls only but no band was observed in all the 62 matched samples of ESCC from Kashmir (Fig. 2).

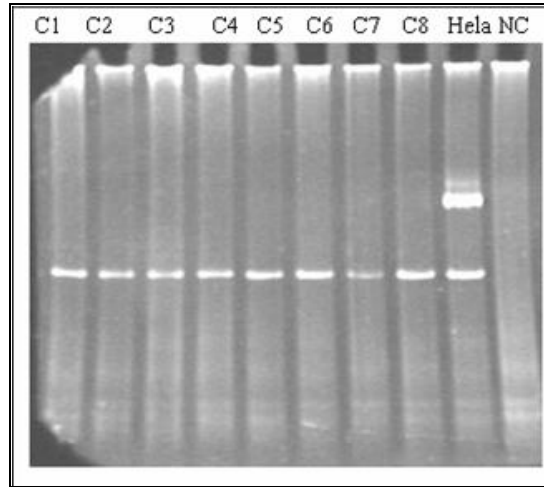


Fig. (2). Electrophoretogram showing PCR based detection of HPV in esophageal cancer samples using PGMY09 and PGMY11 primers with β -globin gene amplification as an internal control. Lanes representing C1, C2, C3 etc. are DNA samples isolated from tumor tissue of patients having esophageal cancer. Here HeLa cell line DNA was taken as positive control and NC represents negative control.

The DNA from HeLa cells, which contain 30-40 copies of HPV 18, yielded intense bands of the correct size while using both primer pairs. In order to check the sensitivity of the method used, DNA from HPV positive cervical carcinoma samples, used as positive control, again yielded the bands of correct size. All the samples showed the good quality of DNA after PCR with β -globin primer as internal control. HPV genotyping of the samples was done using the Line blot assay and of all the patient samples tested, no sample turned out to be positive for HPV (Figure 3). All the 62 samples together with the positive controls, showed satisfactory β -globin amplification in the Line blot assay. HeLa cell line turned out to be HPV 18 type while cervical carcinoma samples P1, P2 and P3 generated the bands corresponding to HPV 45, 52 and 42 types respectively (Fig. 3). The patient samples were negative for all 22 high-risk and 16 low-risk types of HPV, while screened by Line blot assay.

Discussion

While *TP53* mutation has been reported to be the major molecular determinant in the etiology of ESCC from Kashmir, these reports differ in the frequency of p53 mutations and also the exon regions harboring the mutation.^(9,30,31) However all these studies clearly indicate that although *TP53* mutation is important, it alone is not enough to explain the etiopathogenesis of ESCC in the high risk valley of Kashmir. In view of these existing reports we carried a pilot study to analyze genetic alterations in genes involved in the signal transduction events such as β -catenin (studies on N- and K-Ras and B-Raf are in progress). While reports are scanty on the mutational analysis of these genes in the human ESCC, both the animal model studies and the work from *in vitro* cell culture studies point to an involvement of the above mentioned genes in progression of EC. Our consistent observations on PCR-SSCP and direct sequencing make us to strongly believe that mutations in exon 3 of β -catenin have no role in the causation of ESCC in Kashmir. Similar results have very recently been reported from Japan.⁽³²⁾

The role of HPV in ESCC has been studied in many high-risk and low-risk areas of the world.⁽¹⁹⁻³³⁾ Most studies from high-risk areas, such as China and South Africa, have suggested a role of HPV in ESCC, while most studies from low-risk areas have failed to find any association.⁽³⁴⁻³⁵⁾ In a previous study which has been carried out in conjunction with two other endemic geographic regions, New Delhi and Dibrugarh of India, Katiyar. *et al* reported the presence of HPV in 8 (24.2%) of 33 tumors from Kashmir.⁽³¹⁾ Interestingly in contrast to this report, we didn't detect HPV in any of the samples in the present study, though we used two different sets of consensus primers viz., Gp5+ and Gp6+; PGMY09 and PGNY11 in conjunction with reverse Line blot assay to screen for HPV in 68 matched ESCC samples from Kashmir. The sensitivity of our detection system is evident from the fact that DNA from HeLa cells, which contain 30-40 copies of HPV 18 DNA, showed positive amplification for HPV 18. Moreover, the three positive control samples of cervical carcinoma showed positive results corresponding to HPV-45, 52 and 42 types, thus supporting the sensitivity of the method used.

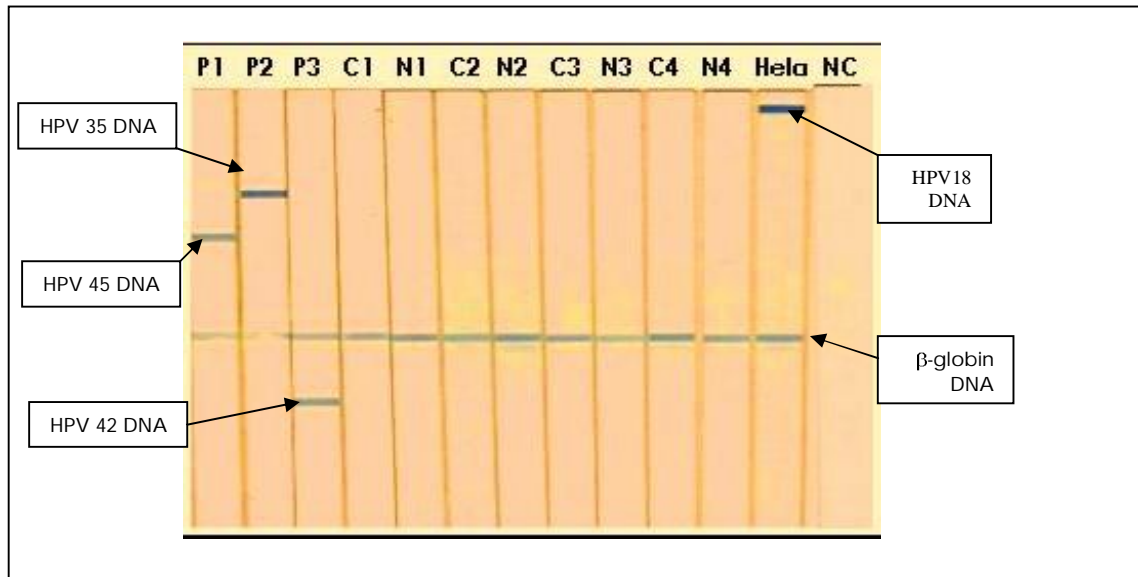


Fig. (3). Representative Line blot picture for detection of HPV in ESCC patients (C1-C4) and control samples (N1-N4). NC represents the negative control. HeLa cell line DNA sample (HeLa) was only positive with respect to HPV-18 and rest all the 62 matched esophageal tumor samples were negative to all 22 high-risk types and 16 low-risk types of HPV strains. P1, P2 and P3 are positive control samples of cervical carcinoma patients which gave a positive test corresponding to HPV 45, 35 and 42 types respectively.

Besides the influence of geographic, environmental and racial differences, many factors can contribute to the considerable variability of the results reported by studies evaluating the association of HPV infection and ESCC, including the specimens analyzed, the HPV detection method used⁽³⁶⁾, interlaboratory variation in performing the same detection method⁽³⁷⁾ and the populations evaluated.⁽¹⁹⁾ The fact that this variability is so pronounced in the studies of HPV and ESCC, in contrast to the studies of HPV and cervical cancer, may imply that any causative association (if at all) must be much weaker and/or less common in the esophageal carcinoma. Prospective cohort studies with large sample size and combination of HPV detection methods may be useful to evaluate the association further.

In conclusion, our results suggest that squamous cell carcinoma of esophagus in Kashmir may arise independent of oncogenic beta-catenin mutations and HPV is unlikely to be an etiologic factor for ESCC in this region.

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