Human papillomavirus 16 E6 is associated with the nuclear matrix of esophageal carcinoma cells

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Abstract

AIM: To explore the etiologic role of HPV infection in esophageal carcinoma, and the association of HPV-16 E6 with the nuclear matrix of carcinoma cells.

METHODS: Two esophageal carcinoma cell lines, EC/CUHK1 and EC/CUHK2, were tested for HPV-16 E6 subgenetic fragment by polymerase chain reaction amplification of virus DNA associated nuclear matrix. RT-PCR and immunocytochemistry were also used to visualize the expression of E6 subgene in the cells.

RESULTS: The HPV-16 E6 subgenetic fragment was found to be present in nuclear matrix-associated DNA, E6 oncoprotein localized in the nucleus where it is tightly associated with nuclear matrix after sequential extraction in EC/CUHK2 cells. It was not detected, however, in EC/CUHK1 cells.

CONCLUSION: The interaction between HPV-16 E6 and nuclear matrix may contribute to the virus induced carcinogenesis in esophageal carcinoma.

Subject headings: Esophageal neoplasms/virology; Esophageal neoplasms/pathology; Tumor cells, cultured; Papillomavirus; human; Nuclear matrix

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INTRODUCTION

Esophageal carcinoma is the most common malignant epithelial neoplasm of the esophagus worldwide. The incidence of esophageal carcinoma reported by WHO has increased many folds in the past 20 years and is apparently still rising. However, the incidence of this disease varies widely among different geographic regions, being remarkably high in certain regions, including the northern parts of China, Iran and South Africa, whereas being remarkably low in Western Europe and North America. The wide geographic variation in incidence of esophageal carcinoma implies a causal role of environmental factors in the pathogenesis of esophageal carcinoma. Epidemiological studies have shown that environmental factors, including tobacco, alcohol, certain dietary products, nutritional deficiency, and other chemical agents are associated with the development of esophageal carcinoma in both high and low risk regions.

Human papillomavirus (HPV) has recently been implicated as an important etiological factor in the development of esophageal carcinoma in high incidence areas. HPV DNA sequences were detected in 24% to 60% of esophageal squamous cell carcinoma in high risk regions. HPV types are small DNA viruses that can be classified as either high risk or low risk group. HPV-16 and 18 is most common in high risk group. In most HPV infected cells, E6/E7 subgenetic fragments of viral genome are retained and integrated in host cell chromosomes. The in vivo and in vitro transforming properties of the virus reside in these two genes. E6 interacts with a tumor suppressor protein P53 and has been shown to target the P53 protein for rapid degradation. E6 expressing cells are therefore unable to express effective levels of P53 protein, resulting in the loss of normal P53 functions within these cells. The ability of the high risk HPV types to interfere with normal P53 function is particularly significant for oncogene activity since alterations of the p53 gene are the most common genetic event detected so far in almost all types of human cancers. E6 also targets other proteins: the focal adhesion protein paxillin and the interferon regulatory factor 3. These data indicate the multifunctions of viral oncoproteins, modifying a multitude of cellular functions.

Nuclear matrix is operationally defined as the nonchromatin component of the nucleus after sequential extraction with non-ionic detergents, nucleases and high salt buffers. It consists of nuclear envelope and inner-fibrous web forming a scaffold for attachment of DNA. Nuclear matrix has been shown to play a fundamental role in nuclear acid metabolism. It has been found to harbor sites for DNA replication, transcription, RNA processing and steroid hormone action. In addition, proto-oncogene products and several viral oncoproteins such as the E B virus nuclear antigen leader protein, adenovirus E1A protein, the simian virus 40 large tumor antigen and HPV E7 protein associate with the nuclear matrix. The involvement of nuclear matrix and its proteins in various nuclear activities has also provided a potential target for anticancer agents and toxicant.

In this study, the association of both HPV-16 E6 gene and E6 protein with nuclear matrix in HPV-16-containing esophageal carcinoma cell line was investigated by PCR, RT-PCR and immunocytochemistry.

MATERIALS AND METHODS

Cell culture

Two human esophageal carcinoma cell lines, EC/CUHK1 and EC/CUHK2, were used. EC/CUHK1 was established from a well-differentiated human esophageal carcinoma and it is HPV negative.
EC/CUHK2 was established from a Chinese male patient with a poorly differentiated squamous carcinoma of the esophagus, the cells contain HPV-16 DNA. The cell lines were cultured in DMEM (Gibco) supplemented with 50 mM L fetal bovine serum (Gibco), 10 units/L penicillin and 100 mg/mL streptomycin (Sigma) at 37°C in humidified 50% CO2 incubator.

Nuclear matrix-associated DNA extraction

The method developed by Fey et al was modified. Briefly, culture cells were trypsinized and resuspended in cytoskeleton buffer (100 mM L KCl, 3 mM L MgCl2, 1 mM L EGTA, 1 mM L Penicillin and 100 mg/mL Streptomycin (Sigma) at 37°C). Following the removal of supernatant, the cells were incubated in cytoskeleton buffer (same as CSK except with 50 mM NaCl instead of KCl) for 30 min at room temperature. The digestion was terminated by addition of cold ammonium sulfate to a final concentration of 0.25 M. After centrifugation, the resulting pellet containing nuclear matrix was digested overnight by proteinase K at 55°C for DNA extraction. The DNA was extracted by phenol-chloroform method and RNA was removed by DNase-free RNase treatment at 37°C. Chromatin was removed by incubation of the pellet with different concentrations (50 and 100 mg/mL) of DNase I and RNase A (100 mg/mL) in digestion buffer (same as CSK except with 50 mM NaCl instead of KCl) for 30 min at room temperature. The digestion was terminated by addition of cold ammonium sulfate to a final concentration of 0.25 M. After centrifugation, the resulting pellet containing nuclear matrix was digested overnight by proteinase K at 55°C for DNA extraction. The DNA was extracted by phenol-chloroform method and RNA was removed by DNase-free RNase treatment at 37°C. After re-extraction with phenol-chloroform, DNA was precipitated with ethanol in the presence of ammonium acetate and stored in Tris-EDTA buffer at 4°C.

Polymerase chain reaction

Purified primers specifying HPV-16 E6 sequences were designed, forming the viral sequence at position nucleotide 82-559 amplifying 477 bp fragments. The primer sequences are 5’-ATGCACCAAAAGAGAACTGC-3’ and 5’-TTACAGCTGCGTTTCTCTAC-3’. PCR was performed in a 25 μl volume of PCR buffer with 1.0 mM MgCl2, 4×dNTPs, primers and sample DNA. The mixture was hot-started to reduce non-specificity. The amplification on reaction was conducted with 30 cycles: 95°C for 1 min; 55°C for 1 min; and 72°C for 1.5 min. HPV-16 plasmid DNA was employed as positive control. The PCR products were finally electrophoresed in 10 g/L agarose gel after ethidium bromide staining.

Total RNA extraction and RT-PCR

Total RNA was extracted by Trizol-chloroform method and precipitated with isopropanol. The RNA pellet was washed with ice-cold 750 μL ethanol, dried and resuspended in DEPC-treated water. The RNA quantity was determined with OD 260 and OD 280. Reverse transcription was performed with random hexamers, reverse transcriptase, RNase inhibitor, dNTPs and 1.5 mM MgCl2 (Perkin Elmer) at 42°C for 45 min. cDNA samples were then subjected to PCR with HPV-16 E6 primer. The amplification condition was the same as PCR.

Nuclear matrix preparations in situ and immunocytochemistry

To obtain whole mount nuclear matrix in situ, the method described by Staufenbiel et al was followed with modifications. The monolayer of culture cells were grown on coverslips for 2 days. After three rinses with PBS at 4°C, the cells were incubated in cytoskeleton buffer, added 4 mM L ribonucleoside vanadyl complexes for 15 min at 4°C. Following the removal of supernatant, the cells were incubated in reticulocyte standard buffer (42.5 mM L Tris-HCl pH 7.4, 8.5 mM L NaCl, 2.6 mM L MgCl2, 1.2 mM L PMSF, 10 mM L Tween 40, 12 mM L sodium deoxycholate, 2 mM L ribonucleoside vanadyl complexes) for 10 min at 4°C. Chromatin was removed by incubation of the cells in DNase I (100 mg/mL) in digestion buffer for 20 min at room temperature and terminated by addition of cold ammonium sulfate to a final concentration of 0.25 M. The nuclear matrix on coverslips was fixed in PBS buffered 100 mM L formalin for 10 min. After blocking the non-specificity, the cells were incubated with anti-HPV-16 E6 (Santa Cruz) antibody. The labeling was then detected by ABC method (Dako) and the result was visualized with DAB substrate. Negative controls were incubated in the absence of primary antibody.

RESULTS

PCR

A specific 477 bp PCR product was amplified by HPV-16 E6 primer in nuclear matrix associated DNA from EC/CUHK2 cell line, whereas DNA from nuclear matrix of EC/CUHK1 cell line showed negative reaction. Positive control using HPV-16 plasmid DNA confirmed the specificity and performance of PCR system. (Figure 1). Different concentrations (50 mg/L and 100 mg/L) of DNase I were employed to elute DNA during nuclear matrix preparation of EC/CUHK2. The residual nuclear matrix associated DNA was subjected to PCR. As shown in Figure 1, 477 bp PCR signal was detected in nuclear matrix associated DNA after 50 mg/L DNase and signal was found weakened in stronger DNase digestion groups.

Figure 1 Results of PCR amplification with HPV-16 E6 primers. 1:100 bp DNA ladder marker. 2: nuclear matrix associated DNA of EC/CUHK1 cells, with 50 mg/L DNase digestion. 3: nuclear matrix associated DNA of EC/CUHK2 cells, with 50 mg/L DNase digestion. 4: nuclear matrix associated DNA of EC/CUHK2 cells, with 100 mg/L DNase digestion. 5: HPV-16 plasmid DNA.

RT-PCR

The expression of HPV-16 E6 in EC/CUHK1 and EC/CUHK2 cell lines were detected in the total RNA level. RT-PCR was conducted using specific HPV-16 E6 primer to detect HPV-16 E6 with 477 bp in length. The samples extracted from EC/CUHK2 showed specific amplification of 477 bp product, but negative results were observed in EC/CUHK1 (Figure 2).

Immunocytochemistry

HPV activity was assayed by anti-HPV-16 E6 oncoprotein antibody. EC/CUHK2 cells were immunolabelled with the antibody. The staining was found concentrated inside the nucleus. E6 oncoprotein distributed diffusely both in the nuclear filamentous structures and in the periphery of the nuclear lamin (Figure 3). In EC/CUHK1 cells, E6 protein immunostaining was negative (results not shown).
with the nuclear matrix of cervical carcinoma cells[33,51-53]. The role of high risk HPV E6 oncoproteins in malignant transformation is supported by their immortalizing and transforming activities in cells in culture. Our findings provided evidence that the viral E6 protein is expressed inside EC/CUHK2 cells after persistent infection and helps efficient immortalization and survival of the infected cells. The interaction of E6 oncoprotein with P53 has been investigated extensively using in vitro systems and in cells[54-57]. The binding of E6 and P53 caused rapid ubiquitin-dependent proteolytic degradation of P53[9,10,58,59]. Cells expressing E6 therefore, have significantly depleted endogenous p53 expression, fail ed to show P53 accumulation following DNA damage and lost normal P53-mediatednegative cell growth control. The E6 targeting of P53 for degradation depends on the ability of the proteins to bind, but not the ability of P53 to form homooligomeric complexes. The subsequent degradation of P53 following the interaction with E6 is dependent on another cell protein, named E6-associated protein (E6-AP).

The E6/E6AP complex functions as a ubiquitin-protein ligase, equivalent to E3 in general ubiquitination pathways, allowing E2 catalysed conjugation of ubiquitin groups to lysine residues on P53[50-52]. The linkage of ubiquitin to P53 in this way serves as a recognition signal for specific protease targeting. In view of the binding of E6 protein and nuclear matrix, we speculate that the E6-AP may be a nuclear matrix protein. It need to be further elucidated.

Since HPV-16 E6 subgenic fragments and oncoprotein are associated with nuclear matrix in esophageal carcinoma cell lines, further studies on the structure and function of these nuclear matrix proteins may provide more information on the relationship between HPV and esophageal carcinoma. It is of great importance to understand the carcinogenesis, and improve the diagnosis and treatment of esophageal carcinoma.

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