Infection of Human Papillomavirus (HPV) and Epstein-Barr Virus (EBV) and p53 Expression in Human Esophageal Carcinoma

Pages with reference to book, From 138 To 142

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Abstract

To clarify the role of high risk human papillomavirus (HPV 16,18 and 33) and Epstein-Barr virus (EBV) infection in esophageal carcinogenesis in relation to expression of mutated p53 antioncogene, we used PCR to amplify DNA sequences of these viruses and immunohistochemistry to detect p53 expression in formaline-fixed, paraffin embedded blocks including 12 normal esophageal and 27 esophageal carcinoma specimens. HPV and EBV DNA were found in 25% and 0% of normal esophageal tissues and in 63% and 7% of esophageal carcinoma specimens, respectively. p53 expression was shown in 59% of esophageal carcinoma specimens only. HPV infection rate was significantly higher in specimens from carcinoma cases as compared with normal esophageal tissue obtained from cases without carcinoma. No correlation was found between p53 expression and/or the presence of viral DNA (HPV/EBV) in regard to the age and sex of the patient, histological grade, histological stage, depth of invasion, lymph node involvement, distant metastasis and the location of the tumors, p53 expression was almost equally distributed between HPV positive and negative carcinoma cases. Our results suggest that most of the esophageal carcinomas are associated with HPV infection and p53 mutations and there is no inverse correlation between HPV and infection and expression of p53 in esophageal carcinoma (JPMA 48:138, 1998).

Introduction

Esophageal cancer shows striking geographical variation in incidence\(^1\) and has poorest prognosis among malignant neoplasms\(^2\). The exact cause of this disease remains unknown despite the substantial amount of data on esophageal carcinoma obtained in recent past\(^3\). Extensive epidemiological and experimental studies have suggested several risk factors such as cigarette smoking, intake of excess alcohol, hot foods, some specific nutritional deficiencies including vitamin and mineral, and some chemical substances in food especially nitrosamines and their precursors\(^1\). Like anogenital and other aerodigestive cancers sufficient data is now available to implicate human papillomaviruses as one of the etiological agent in esophageal carcinogenesis\(^1,3\). Similarly, at molecular level besides other genetic factors it has been found that alteration of p53 which is one of the tumor suppressor gene are quite common in this malignancy\(^4\). Recently in vitro studies showed that oncoprotein E6 encoded by high risk HPV (type 16 & 18) binds to normal cellular p53 protein and results in selective degradation of the latter, resulting in loss of the negative control normally exerted by p53\(^5\). Analysis of cervical cancer cell lines\(^6\) and cervical carcinoma specimens\(^7\) have substantiated these experimental facts, however, we could not observe this correlation in our recent studies involving laryngeal and gastric carcinomas\(^8,9\). Therefore we decided to study a series of esophageal carcinomas both for high risk HPV and p53 mutations. Moreover, we also studied role of EBV for the first time in this malignancy, keeping in view the recent reports of presence of this virus in some cases of AIDS associated esophageal ulceration\(^1\).
Materials and Methods

A variety of esophageal carcinoma specimens from 27 (20 male and 7 female) patients with mean age of 66.2 (ranging from 46 to 80) years were obtained from the Pathology Laboratory of Fukui Medical School Hospital, Fukui, Japan. Staging, grouping and histological classification were based on the Guide lines for the clinical and pathological studies on carcinoma of the esophagus established by Japanese Society for Esophageal Diseases\textsuperscript{10}. Twelve specimens of normal esophagus (8 male and 4 female) obtained at autopsy from patients who had died of causes other than malignancy and immunocompromised diseases, were also examined for comparison. The mean ages of the patients with out gastric carcinoma were 64.6 (ranging from 44 to 82) years. All tissues had been fixed in 10\% buffered formaldehyde and embedded in paraffin. The paraffin blocks used in this study were stored at room temperature for 1-4 years.

Cell lines and Recombinant HPV DNA

Two cell lines (Raji and Daudi) containing EBV, obtained from Japanese Cancer Research Resources Bank, were maintained in vitro according to the instructions of the supplier. DNA from these two cell lines and recombinant viral DNAs of HPV 16, 18 and 33 were used as target DNA for PCR amplification with their specific primers in control experiments as described previously\textsuperscript{9,11}.

DNA Extraction

DNA extraction was carried out according to the previously described methods for formaldehyde-fixed, paraffin embedded tissues\textsuperscript{9,11,12}. The genomic DNA from the cell lines used as positive control was prepared according to the established procedures\textsuperscript{13}.

Polymerase Chain Reaction (PCR)

PCR was performed as described before\textsuperscript{9,11,12}. Twelve ul of the PCR products were routinely checked for amplified DNA on 5\% polyacrylamide gel.

Synthetic Oligonucleotides

All HPV, beta-globin gene and EBV primers and probes were synthesized on an ABI 381A DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). The sequences for HPV and beta-globin gene primers and HPV probes were same as described before.\textsuperscript{1} The sequences of primers and probes for EBNA 1 gene of EBV were modified from the previously published sequences and these were as follows: sense primer, 5-GGACCTCAAAGAAGGGGG, antisense primer; 5-CTGGTCTTCCGCTCTCTCGT, probe, 5-TAACACTGGGACGGAGGACG GGAAGAGGGACG. The size of the amplified fragment by this set of primers is 76 bp. The oligonucleotide HPV and EBV probes were labeled by phosphopylation with -32p\] ATP (specific activity >5000 Ci/mmool; 1 Ci = 37 GBq) (Amersham, Tokyo, Japan) and T4 polynucleotide kinase (Takara Biochemicals, Tokyo, Japan). They were routinely purified by spin-column chromatography through 1 ml of Sephadex G-25 Medium (Pharmacia, LKB Biotechnology, Uppsala, Sweden). This procedure gave probes with specific activity of about cpm/ug.

Gligonucleotide Probe Hybridization

For detection of HPV and EBV, the preparation of filters and protocols for prehybridization, hybridization and washes were same as described\textsuperscript{9,11}. After the washes, the filters were dried and exposed to XAR film (Eastman Kodak Company, Rochester, NY) at -70°C for 1-12 h. Immunohistochemistry p53 staining was performed using a polyclonal antibody to p53 (CM1) and a 3-layered immunoperoxidase method as described before\textsuperscript{8,12,14}. The intensity and pattern of p53 immunostaining were tabulated according to criteria reported by Midgley et al\textsuperscript{14}. Briefly it was as follows: ++++, more than 70\% of tumor nuclei were intensely stained; ++, between 10-70\% of tumor nuclei were intensely stained; +, less than 10\% of tumor nuclei intensely stained and/or a variable number of tumor nuclei faintly stained; -, tumor nuclei unstained.
Statistical analysis
Statistical analysis was performed using all parameters separately and in a multivariate analysis. Two-sided P values of less than 0.05 were considered statistically significant.

Results
Twenty seven esophageal carcinoma samples from the same number of patients were screened for the presence of high-risk HPV and EBV DNA. The specimens were also immunohistochemically stained with p53 specific polyclonal antibody (CM 1). These results in relation to clinicopathological features for prognosis of esophageal cancer are summarized in Table 1.

### Detection of high risk HPV DNA
Of 27 cases 17 (63%) were positive for at least one type of high-risk HPV. HPV 16, 18 and 33 DNA was detected in 3 (18%), 4 (24%) and 16 (94%) cases, respectively (Figure 1, b,c,d). Six cases harbored more than one type of HPV DNA. Only 3 (25%) of 12 normal esophageal tissues were found to harbor HPV 33 DNA. HPV infection was significantly increased in esophageal carcinoma specimen as compared with nonnal esophageal tissue ($\chi^2=4.79 \ p<0.05$). No correlation was found between the presence of HPV DNA and any of the clinicopathological features for prognosis shown in Table 1.

### Detection of EBV DNA
EBV DNA was found in 2 (7%) of 27 carcinoma cases (Figure 1).

One of the case revealed simultaneous presence in the gastric carcinoma. There was no correlation between the presence of EBV DNA and any of clinicopathological features for prognosis of esophageal carcinoma. None of the normal esophageal tissues were found to contain EBV DNA.

Immunohistochemistry with p53 antibody p53 was detected in 16 (59%) of 27 cases. Seven cases showed positivity confined to <10%(+) of tumor cells, 5 cases 10-70%(++) of tumor cells and int
remaining 4 cases more than 70% (±++) of tumor cells were positive for p53. In most of the cases the immunoreaction was localized in nucleus of the neoplastic cells (Figure 2).

None of the normal esophageal epitheliurn adjacent to the neoplastic tissue and 12 normal esophageal tissue was found positive for p53. Similar to other two parameters the expression of p53 also did not correlate with any of the clinicopathological feature for prognosis of esophageal carcinoma.

**Correlation between HPV DNA/EBV DNA and expression of p53**
There was no positive correlation among these parameters (Table II).
Moreover, p53 expression was almost equally distributed between JJPV positive and HPV negative carcinoma cases and 3 of the 4 cases revealing strong positivity (+++) with CM-i antisera were positive for HPV DNA.

**Discussion**

Like cervical carcinoma, now sufficient data has accumulated to suggest etiological role of HPV in esophageal carcinogenesis. Several studies have demonstrated involvement of HPV in benign and malignant lesions of esophagus. Since first report in 1982 by Syrjanen, who found HPV related histological changes in 40% of patients with esophageal carcinoma, HPV now has been demonstrated in these lesions by immunohistochemical and DNA hybridization techniques. Previous studies have reported presence of high frequency of HPV DNA in esophageal carcinomatous lesions from China, South Africa and Alaska natives. However, recently reports from Japan and France have also implicated HPV role in esophageal squamous cell carcinoma. In our present study both normal and neoplastic esophageal specimens were found to harbor high risk HPV DNA. However, presence in neoplastic lesion (63%) was significantly higher than normal esophageal tissues (25%). This is in contrast to a report from Japan by Yasuhi et al who could demonstrate presence of high risk HPV in only 3 of 45 (6.7%) cases, however, in another study from a different area in Japan by Furihata et al, HPV was found in 39% of esophageal carcinomas by in situ hybridization. This kind of discrepancy although could not be explained, the variation in methodology may be an important factor in this regard.

EBV has been implicated in lymphoid and nasopharyngeal malignancies, besides its more recent role in gastric carcinomas. It has also been reported in some cases of AIDS associated esophageal ulceration. We could find presence of EBV DNA in only 2 esophageal carcinomas and these two cases has no distinguishing characteristics as compared with the cases found negative. So at least in our

<table>
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series EBV does not seem to be an important factor, although total number of cases analyzed is not sufficient to make such conclusion.

Like other malignancies p53 mutations are quite common in esophageal carcinoma. Previous studies have demonstrated presence of p53 mutation in up to 50% of squamous cell carcinomas and 67% of adenocarcinomas. We found 59% of esophageal squamous cell carcinomas positive for p53 expression by immunohistochemical method, which previously has been shown to be closely correlated with the presence of p53 mutations. Interestingly most of the cases (11 of 16) which were found positive for p53 also harbored high HPV DNA. Previous studies of cervical cell lines and a series of cervical carcinomas by the same group of investigators has shown the inverse correlation between the presence of high risk HPV and the presence of somatic mutation of p53. Moreover, Furihata et al has also demonstrated same relation in their study involving esophageal carcinomas. However, like our previous studies involving laryngeal, gastric and female genital carcinomatous lesions, we could not observe this inverse correlation even in esophageal malignancy. Moreover, 3 of 4 cases strongly positive (+++) for p53 expression were positive for HPV DNA. Previously, we have suggested that our results may be explained by presuming secondary infection with HPV in these carcinoma cases which already had p53 mutations or due to heterogeneity of the tumor cells HPV infection may be confined to those clones of tumor cells which are lacking p53 mutations. In a recent study Lam et al demonstrated positive p53 staining in 100% of the human papilloma virus positive penile carcinomas. Moreover, now it has been experimentally clarified that only binding of high risk HPV E6 to the core of p53 leads to its degradation, but similar binding to C-terminal region has no effect on the integrity of p53. Likewise Suzuki et al showed that HPV 16 immortalized HLEC cell line had acquired p53 mutation during the immortalization process.

We could not find any correlation between the presence of HPV DNA/EBV DNA or p53 mutations and any of the clinicopathological features for prognosis shown in Table I. However, this may be due to relatively small number of cases analyzed in the present study.

Our results suggest that most of the esophageal carcinomas are associated with HPV infection and p53 mutations and there is no inverse correlation between HPV and infection and expression of p53 in esophageal carcinoma.

Acknowledgements

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References