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

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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 ins pecimens 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 IIPV 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 epiderniological and experimental studies have suggested several risk factors such as cigarette smoking, intake of excess alcohol, hot foods, some specific nutritional deficiencies includingvitamin 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 lazyngeal 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 and7 female) patients with mean age of 66.2 (ranging from 46 to SO) 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.patho!dgic..studies on carcinoma of the esopbagus established by Japanese Society for Esophageal Diseases[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 wre 64.6 (ranging from 44 to 82) years. All tissues had been fixed in 10% buffered formaline and embedded in paraffin. The paraffin blocks used in this stud 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 instmctions of the supplier. DNA from these two cell imes and recombinant viral DNAs of HPV 16, 18 and 33 were used as targetDNA forPCR amplification with their specific primers in control experiments as described previously[9,11].

DNA Extraction

DNA extraction was carried out according to the previously described methods for formaline-fixed, paraffin embeddedtissues[9,11,12]. The genomicDNAfmmthe celilines used as positive control was prepared according to the established procedures[13].

Polymerase Chain Reaction (PCR)

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

Synthetic Oligonucleotides

All HP V, 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.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 priemr, 5-GGACCTCAAAGAAGAGGGGG, antisense primer; 5-CTGGTCTTCCGCCTCCTCGT, probe, 5-TAACCATGGACGAGGACG GGGAAGAGGACG.

The size of the amplified fragmentby this set ofprimeris 76 bp. The oligonucleotide HPV and EBV probes were labeled by phosphoiylation with -32p] ATP (specific activity >5000 Ciimrnol; I Ci= 37 GBq) (Amersham, Tokyo, Japan) and T4 polynucleotide kinase (Takara Biochemicals, Tokyo, Japan). They were routinely purified by spun-column chromatography through I ml of Sephadex G-25 Medium (Pharmacia, LKB Biotechnology, Uppsala, Sweden). This procedure gave probes with specific activity of about cpm/ug.

Gligonucieotide Probe Hybridization

For detectionof HPV and EB V, the preparation of filters and protocols for prehybridization, hybridization and washes were sme as described9'11. After the washes, the filters were dried and exposed to XAR film (East-man Kodak Company, Rochester, NY) at -70C 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[8,12,14]. The intensity and pattern of p53 immunostaining were tabulated according to criteria reported by Midgley et al[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 perfonned using all parameters sepamtely and in a multi variate 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 clinicopathologica1 eatures for prognosis of esophageal cancer are summarized in Table I.

Table I. Clinicopathological data in relation to presence/absence of Human Papillomavirus (HPV) and/or Epstein Barr-virus (EBV) DNA and expression of p53 in esophageal squamous cell carcinoma.

Patient no.	Age	Sex	Stage	Depth of invasion	Lymph node metastasis	Organ metastasis	Histological type	Vascular invasion	HPV	EBV	p53
1	76	F	I	mp	n0	MO	Mod	ly- v-	-	+	-
2	70	M	IV	a3	n4	MO	Well	ly- v-	-	+	-
3	67	M	IV	a3	n2	MO	Mod	ly- v-	-	-	+++
4	63	M	IV	a2	n4	MO	Well	ly- v-	-	-	+
5	62	M	IV	sm	n3	MO	Poor	ly- v-	33	-	++
6	61	F	IV	a2	n3	MO	Poor	ly+ v-	16,33	-	++
7	61	F	IV	mp	n3	MO	Well	ly- v-	-	-	-
8	53	M	IV	a3	n2	MO	Well	ly+ v+	-	-	-
9	80	M	III	a2	n2	MO	Well	ly- v-	33	-	+
10	66	M	II	a1	nO	MO	Well	Ly+ v-	-	-	++
11	57	M	III	a2	nO	MO	Mod	ly+ v+	16,33	-	+
12	46	M	0	sm	nO	MO	Mod	ly+ v-	-	-	++
13	60	F	III	mp	n2	MO	Poor	ly+ v-	-	-	+
14	69	M	III	a2	n2	MO	Mod	ly+ v+	33	-	-
15	77	M	III	a1	n2	MO	Mod	ly+ v+	16,33	-	+++
16	64	M	IV	a2	n3	MO	Well	ly+ v+	33	-	-
17	52	F	IV	a1	n4	MO	Poor	ly+ v+	33	-	-
18	73	M	II	a1	n0	MO	Poor	ly- v+	33	-	+++
19	65	M	IV	a1	n3	M1	Mod	ly+ v+	18,33	-	+
20	52	M	IV	a3	n3	M1	Well	ly+ v+	33	-	-
21	72	F	I	mp	nO	MO	Carc.	ly- v-	18	-	-
22	71	M	IV	a1	n3	MO	Mod	ly+ v+	33	-	+
23	52	M	III	a1	n2	MO	Mod	ly+ v+	33	-	-
24	71	M	I	mp	nO	MO	Mod	ly+ v+	33	-	+
25	73	M	I	sm	nO	MO	Well	ly+ v-	18,33	-	+++
26	52	F	II	a1	n1	MO	Mod	ly+ v+	-	-	+
27	69	M	IV	a1	n3	MO	Mod	ly+ v-	18,33	-	-

sm: invasion to submucosa; mp: invasion into muscular layer; a1: invasion reaching the adventitia; a2: definite invasion to adventitia; a3: invasion into the neighbouring structures; nO: no metastasis; n1 metastasis to group 1 of the regional lymph nodes; n2: metastasis to group 2 of the regional lymph nodes; n3: metastasis to group 3 of the regional lymph nodes; n4: metastatic lymph nodes beyond group 3; MO: metastasis negative; M1: metastasis to distant organ; Well: well differentiated squamous cell carcinoma (SCC); Mod: moderately differentiated SCC; Poor: poorly differentiated SCC; ly-: no invasion of lymphatic vessels in histological sections; ly+: invasion of lymphatic vessels in histological sections; v-: no invasion of blood vessels in histological sections, v+; invasion of blood vessels in histological sections Note: stage grouping depth of invasion, lymph node metastasis, organ metastasis and histological types were based on the Guide Lines for the Clinical and Pathological Studies on Carcinoma of the Esophagus in Japan.

Detection of high risk HPV DNA

Of 27 cases 1 7(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 ($x2=4.79$ $p<0.05$). No correlation was foundbetween 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).

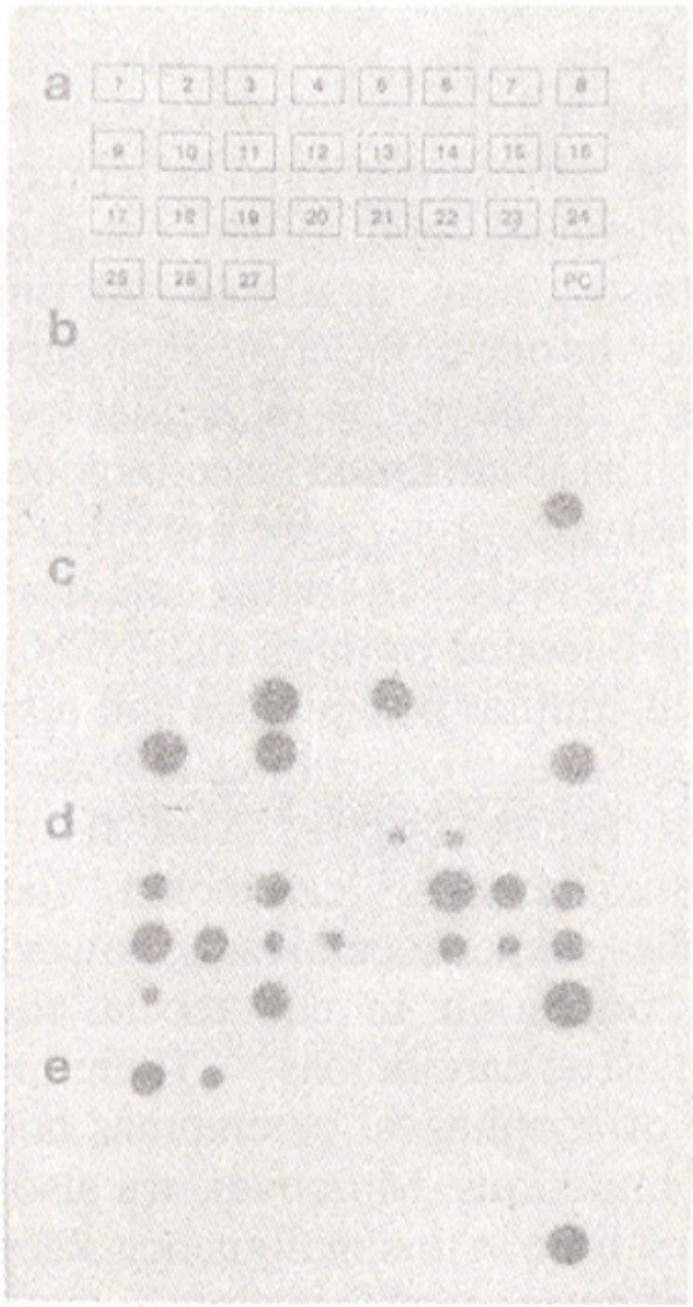

Figure 1. Detection of HPV 16, 18, 33 and EBV sequences in esophageal carcinoma specimens. Twenty seven esophageal carcinoma specimens and cloned HPV 16, 18, 33 and EBV DNA as a positive control were amplified, spotted onto nylon filter and hybridized with a ^{32}P-labeled oligonucleotide probe specific for HPV 16 (b), HPV 18 (c), HPV 33 (d) and EBV (e). The arrangement of the specimens and positive control (P) in autoradiogram is as in the reference grid (a).

One of the case revealed simultaneous presence in the gastric carcinoma. There was no correlation between the presence of EB VDNA 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 1O-70%(++) of tumor cells and inthe

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).

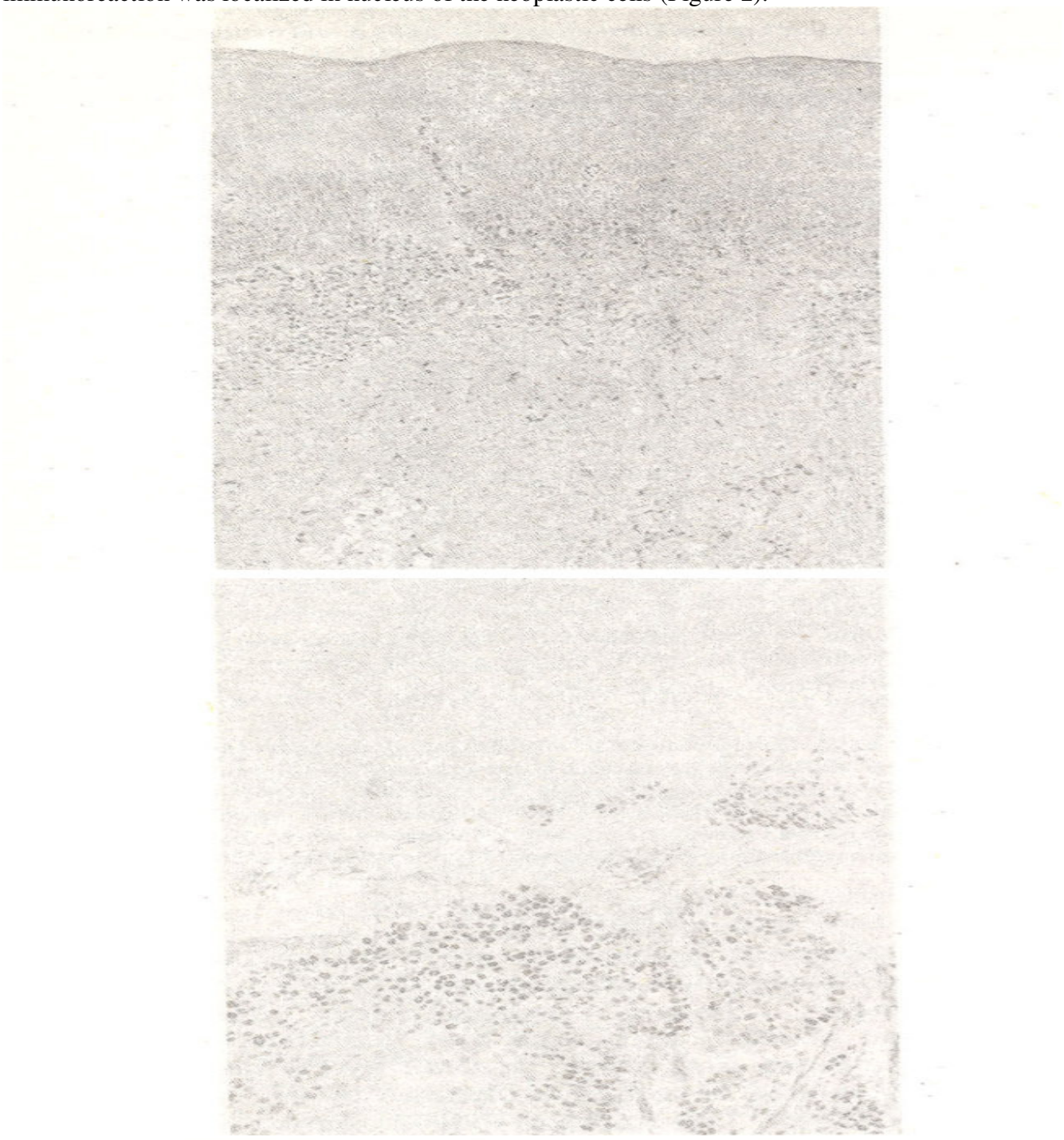

Figure 2. Immunohistochemical analysis of an esophageal squamous cell carcinoma. (a) Section stained with H & E: (b) the same section stained with CM-1 polyclonal anti-serum, demonstrating intense nuclear staining (+++) for p53 protein, which is exclusively confined to neoplastic cells. Scale bar: 60 um.

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 IIPV DNA/EBV DNA and expression of p53

There was no positive correlation among these parameters (Table II).

Table II: Correlation between HPV and EBV infection and expression of p53 in 27 esophageal carcinomas.

HPV	EBV	p53	Number of cases
+	+	+	0
+	+	-	0
+	-	+	10
+	-	-	7
-	+	+	0
-	+	-	2
-	-	+	6
-	-	-	2
Total	17 (80%)	2(6%)	16(66%)	27(100%)

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[1]. Several studies have demonstrated involvement of HPV in benign and malignant lesions of esophagus[1]. Since first report in 1982 by Syrjanen[15], who found HPV related histological changes in 40% of patients with esophageal carcinoma, HPV now has been demonstarated in these lesions by immunohistochemical and DNA hybridization techniques[1]. Previous studies have reported presence of high frequency of HPV DNA in esophageal carcinomatous lesions from China, South Africa and Alaska natives1. However, recently reports from Japan[16,17] and France[18] 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[16] who could demonstrate presence of high risk HPV in only 3 of 45 (6.7%) cases, however, in another study from a different area inJapanby Furihata et al[17], 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[19,20]. It has also been reported in some cases of AIDS associated esophageal ulceration[1]. We could fmd presence of EBV DNA in only 2 esophageal carcinomas and these two cases has no distinguishing characteristics as comapred with the cases found negative. So at least in our

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[4]. Previous studies have demonstrated presence of p53 mutation in upto 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[21]. 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[6] and a series of cervical carcinomas[7] 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[17] has also demonstrated same relation in their study involving esophageal carcinomas. However, like our previous studies involving laryngeal[8], gastric[9] and female genital carcinomatous lesions[22], we could not observe this inverse correlation even in esophageal malignancy. Moreover, 3 of 4 cases strongly positive (+±+) for p53 expressionwere positive for HPV DNA. Previously[8,9]. we have suggested that our results may be explained by presuming secondaiy.infection with HPV in these carcinoma cases which already had p53 mutations or due to heterogeneity of the tumor cells HPV infecion may be confined to those clones of tumor cells which are lacking p53 mutations. In a recent study Lam et al[22] demonstrated positive p53 staining in 100% of the human papilloma vims 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[23]. Likewise Suzuki et al[24] shoed that HPV 16 £7 immortalized HLEC cell line had acquired p53 mutation during the immortalization process.

We could not find any correlation between the presence of HPV DNAIEBV 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 muations and there is no inverse correlationbetweenHPVand infection and expression of p53 in esopahgeal carcinoma.

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