

Absence of human papillomavirus genomic sequences detected by the polymerase chain reaction in oesophageal and gastric carcinomas in Japan

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Abstract

Aims—To estimate the occurrence of human papillomavirus (HPV) infection in oesophageal or gastric carcinomas in patients in Japan.

Methods—103 oesophageal and 99 gastric carcinomas were investigated by polymerase chain reaction (PCR) assays using two consensus (targeting either the L1 or the E6-E7 regions) and two type specific (type 16 and 18) primer sets. A β globin gene specific primer set was also applied to examine the quality of the extracted DNA.

Results—Amplification of β globin gene was clearly visible in 92 (89.3%) of the 103 oesophageal, and 89 (89.8%) of the 99 gastric carcinoma specimens. However, the entire series of tumour DNA was negative for HPV sequences by PCR assay using all four primer sets.

Conclusion—HPV is not likely to be involved in oesophageal or gastric tumorigenesis in Japanese patients.

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Keywords: human papilloma virus; oesophageal carcinoma; gastric carcinoma

Human papillomaviruses (HPVs), members of the papovavirus family, are closed circular double stranded DNA viruses about 7.9 kilobases long that have been reported to demonstrate approximately 60 different genotypes.^{1,2} HPVs are subclassified into high risk (HPV-16, HPV-18, HPV-31), and low risk (HPV-6, HPV-11) categories on the basis of their association with malignant progression.³ The E6 protein of high risk HPV types has been shown to bind to the tumour suppressor p53 protein and the E7 protein binds to Rb protein,^{4,5} suggesting a possible role of HPV infection in tumour development. Earlier studies have demonstrated the presence of HPV genomes in several human cancers, particularly squamous cell tumours of the uterine cervix,⁶ skin,⁷ conjunctiva,⁸ and upper respiratory tract.^{9,10}

Oesophageal carcinomas have a distinct geographic distribution with a high prevalence in certain regions of Asia, Africa, Iran, France, and South America,¹¹ and marked differences in incidence of about 300-fold between different areas or countries.¹² Several risk factors have been suggested, such as cigarette smoking, excessive intake of alcohol and hot foods,

and specific nutritional deficiencies.¹³ Some studies have demonstrated the presence of HPV DNA in oesophageal carcinoma samples using in situ hybridisation, polymerase chain reaction (PCR) and Southern blot techniques^{14,15}; other reports documented lack of HPV sequences in tumour DNA investigated by similar methods.^{16,17} The question of whether HPV infection plays a role in the genesis of oesophageal carcinomas in some populations, especially in high incidence areas, remains open.

The incidence of gastric carcinomas has also been found to demonstrate marked geographic variation. Although the majority of HPV related malignant tumours are squamous cell carcinomas, a few studies have demonstrated an association of HPVs with non-squamous cell carcinomas, such as adenocarcinomas of the uterine cervix¹⁸ and colon,¹⁹ and transitional cell carcinomas of the urinary bladder.²⁰

The present study investigated the occurrence of HPV infection in oesophageal and gastric carcinomas arising from the upper digestive tract of Japanese patients by PCR assay using two consensus (L1 and E6-E7 regions) and two type specific (type 16 and 18) primer sets.

Patients and methods

CASES

One hundred and three oesophageal and 99 gastric carcinoma cases were selected from the patient charts of the authors' institution from 1989 to 1994. All samples were surgically resected, and conventionally fixed in 10% formalin, and paraffin embedded. Histological classification was performed according to the criteria of the Japanese Society for Oesophageal Disease (1992)²¹ or the Japanese Research Society for Gastric Cancer (1993).²² Lesions were further classified into early and advanced categories, in accordance with the depth of tumour invasion: to the mucosa and/or the submucosa in the former case, and into or through the muscularis propria in the latter.

DNA EXTRACTION

Several serial 10 μ m paraffin sections were stained with 1% methyl green solution, tumour cells were then scraped off under the microscope, carefully avoiding contamination with areas of more than 25% to 30% normal cells. Tissue samples were treated with lysis buffer containing 100 μ g/ml proteinase K (Merck, Darmstadt, Germany) at 48°C for 48 hours.

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Table 1 Primer sequences used for the polymerase chain reaction

	Target region	Primer sequence	Source	Approximate product size (base pairs)		
Consensus	L1	L1C1	5'-CGTAAACGTTTTCCCTATTTTTT-3'	Yoshikawa <i>et al</i> ²⁴	250	
		L1C2	5'-TACCCTAAATACTCTGTATTG-3'			
	E6-E7	pU-1M	5'-TGTCAAAAACCGTTGTGTCC-3'	Fujinaga <i>et al</i> ²⁵		240 (Type 16) 270 (Type 18)
Type specific	Type 16 (E6)	pU-2R	5'-GAGCTGTCGCTTAATTGCTC-3'	Shimada <i>et al</i> ²⁶	140	
		p16-1	5'-MGGGCGTAACCGAAATCGGT-3'			
	Type 18 (E6)	p16-2R	5'-GTTTGCAGCTCTGTGCATA-3'	Bloss <i>et al</i> ²⁷		109
		p18-1	5'-ACAGTATTGGMCTTACAGA-3'			
β globin		p18-2R	5'-GAATMAAATCTATACATTT-3'	Coates <i>et al</i> ²³	355	
		BGLO3	5'-ATGGTGCACCTGACTCCTGAGG-3'			
		BGLO4	5'-GCCATCACTAAAGGCACCGAGC-3'			

DNA extraction was performed by phenol/chloroform treatment and precipitation overnight with ethanol. The DNA pellets were dissolved in distilled water and their concentrations determined by spectrophotometry. The quality of the DNA extracted was confirmed with a β globin gene specific primer pair amplifying a 355 base pair fragment.²³

POLYMERASE CHAIN REACTION

Aliquots of DNA (50 ng) were used as templates in a reaction volume of 10 μ l containing 1 μ M each primer and 0.5 U Taq DNA polymerase (Takara, Shiga, Japan) (table 1).

The consensus primers targeting the L1 region (L1C1/L1C2)²⁴ are able to detect nine types of HPV (6, 11, 16, 18, 31, 33, 42, 52, and 58). The PCR analysis entailed 40 cycles of 30 seconds at 94°C, two minutes at 48°C, and two minutes at 72°C. The other consensus primers targeting the E6 and E7 open reading frames (pU-1M/pU-2R)²⁵ can amplify six types of HPV (16, 18, 31, 33, 52b, and 58). The PCR analysis entailed 40 cycles of 30 seconds at 94°C, two minutes at 55°C, and two minutes at 72°C. For determination of HPV subtypes, a restriction fragment length polymorphism assay was performed with digestion of the PCR products with appropriate restriction enzymes including *Rsa*-I, *Dde*-I, *Hae*-III, and *Ava*-II. The type specific primers (p16-1/p16-2R,²⁶ and p18-1/p18-2R²⁷) targeting the E6 open reading frame were able to amplify HPV-16 and HPV-18, respectively. The PCR reaction entailed 40 cycles of 30 seconds at 94°C, two minutes at 55°C, and two minutes at 72°C.

To check for false positives, water instead of DNA was included with each amplification. As positive controls, cervical carcinoma cases confirmed to carry HPV types 16 and 18,⁶ were used. PCR was carried out in duplicate or triplicate.

The sensitivity of the PCR assay using each primer set was examined with serial dilutions of subcloned HPV type 16 or 18 DNA containing 50 ng normal genomic DNA of human liver tissues.

Results

Amplification of β globin gene resulted in clearly visible products in 92 (89.3%) of the 103 oesophageal carcinoma specimens, and 89 (89.8%) of the 99 gastric carcinoma specimens, providing 181 carcinoma specimens for examination of HPV DNA detection.

At least 0.001 pg of subcloned HPV DNA containing 50 ng normal human genomic DNA could be detected by the PCR procedure using four primer sets (fig 1).

The 92 oesophageal tumours were histologically classified as 22 (23.9%) well differentiated, 25 (27.2%) moderately differentiated,

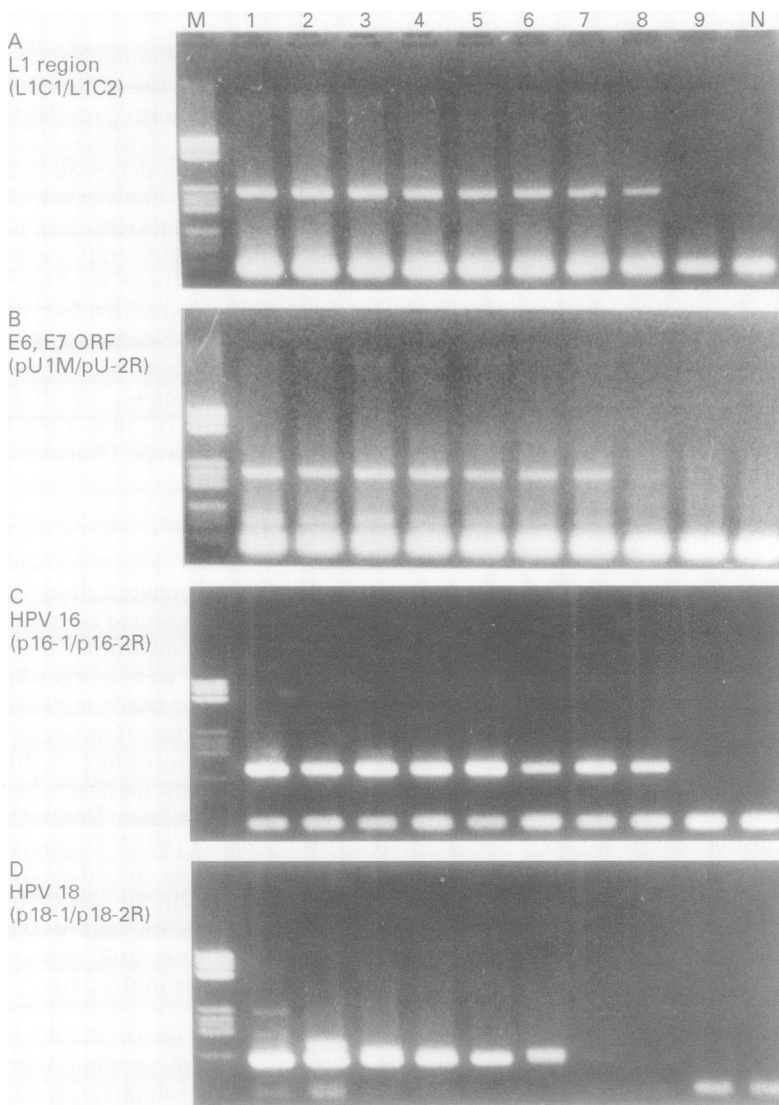


Figure 1 Sensitivity of PCR to amplify HPV DNA. Serial dilution series of subcloned HPV type 16 and 18 DNA containing 50 ng normal human genomic DNA were amplified using primer pairs, L1C1/L1C2 (A), pU1M/pU-2R (B), p16-1/p16-2R (C), and p18-1/p18-2R (D). Concentrations of HPV DNA: lane 1, 1000 pg; lane 2, 100 pg; lane 3, 10 pg; lane 4, 1 pg; lane 5, 0.1 pg; lane 6, 0.01 pg; lane 7, 0.001 pg; lane 8, 0.0001 pg; lane 9, 0.00001 pg. M, molecular markers (DNA molecular markers V, Boehringer Mannheim/Yamanouchi); N, negative control.

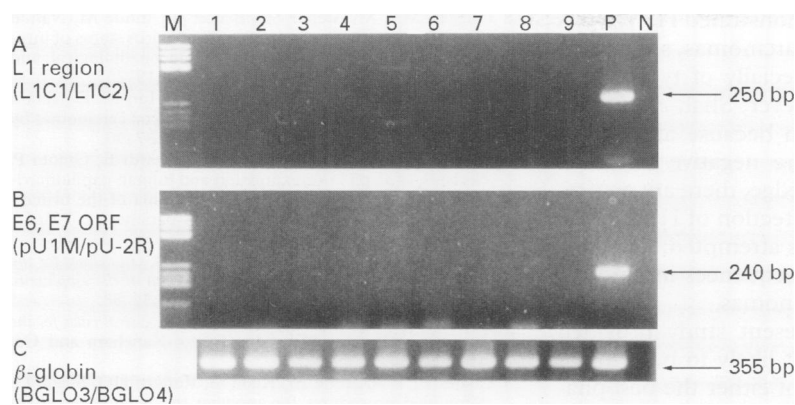


Figure 2 Results of PCR using two consensus primer sets, targeting regions L1 (A) or E6-E7 open reading frames (ORF) (B). The positive control (P, HPV type 16 within cervical carcinoma case) is clearly amplified by both primers, while all tumour DNA (lanes 1-5, oesophageal carcinoma cases, lanes 6-9, gastric carcinoma cases) is negative. Appropriate molecular size products for the β globin gene are consistently present in tumour and positive control samples (C). M, molecular markers (DNA molecular markers V, Boehringer Mannheim/Yamanouchi); N, negative control.

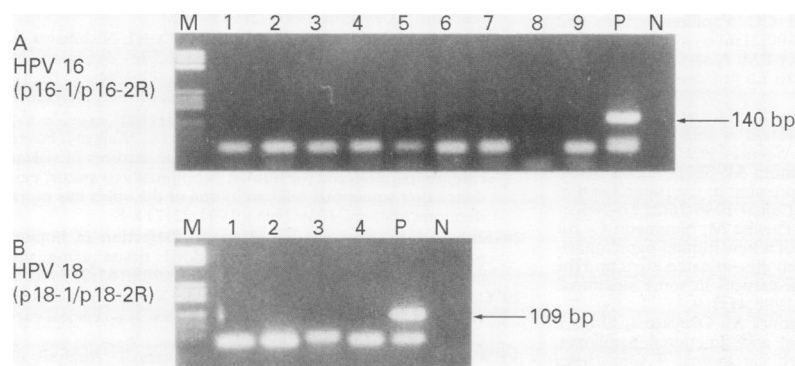


Figure 3 PCR using type specific primers. (A) PCR for HPV type 16. Lanes 1-5, oesophageal carcinoma cases, lanes 6-9, gastric carcinoma cases. P, positive control (HPV type 16 within a cervical carcinoma case), N, negative control. (B) PCR for HPV type 18. Lanes 1 and 2, oesophageal carcinoma cases; lanes 3 and 4, gastric carcinoma cases, P, positive control (HPV type 18 within a cervical carcinoma case); N, negative control. M, molecular markers (DNA molecular markers V, Boehringer Mannheim/Yamanouchi); N, negative control.

and 45 (48.9%) poorly differentiated squamous cell carcinomas, including 48 at early, and 44 at advanced stages. Eighty nine gastric lesions were classified as 21 (23.4%) well differentiated, 23 (25.8%) moderately differentiated, and 45 (50.6%) poorly differentiated adenocarcinomas, 43 in early and 46 in advanced stages.

HPV genomic sequences could not be identified in any of the 92 oesophageal or 89 gastric tumour DNAs using consensus primers for either the HPV L1 region or the E6 and E7 open reading frames (fig 2). Type specific primers for HPV-16 and HPV-18 also failed to amplify the HPV DNA (fig 3). The positive controls always gave products showing the expected molecular size and negative controls were consistently negative (figs 2 and 3).

Discussion

Detection of HPV genomic sequences is strongly dependent on the sensitivity of the technique used: PCR can detect as few as 10 to 20 copies of HPV DNA²⁸; in situ hybridisation (ISH) can detect 50 to 200 viral DNA copies. Although ISH allows the anatomical location of tumour cells demonstrating HPV infection to be distinguished, non-specific and false positive staining may occur.¹⁷ It has been

reported that in PCR assays, detection of high risk HPV types by primers within the E6 gene region is more sensitive than the consensus primer sets, as the E6 and E7 genes, responsible for transforming ability, are highly conserved in most carcinomas, even when the virus is integrated into the host genome.^{29, 30} In the present study, we applied a comprehensive approach to detection using two consensus primers and two type specific primer sets. The PCR system with consensus primers for either the L1 region (L1C1/L1C2) or the E6 and E7 open reading frame (pU-1M/pU-2R) can detect 0.1 to 1.0 copy of the HPV genome per cell,^{24, 25} and the type specific primers (p16-1/p16-2R) can amplify 10^{-5} to 10^{-6} viral copies per cell by combination of PCR and dot blot hybridisation²⁴; these values are supported by our results for the sensitivity of HPV DNA detection on PCR assay using same primer sets. Thus, our detection assay was of relatively high sensitivity; however, it is possible that some HPV positive specimens with extremely low levels of HPV DNA could have been missed.

A possible role for HPV in oesophageal carcinogenesis was initially suggested by the finding of morphological koilocytotic changes of oesophageal mucosa in patients with oesophageal squamous cell carcinomas.³¹ Recent molecular biological analyses demonstrated that positive rates for the presence of HPV genomes in oesophageal carcinomas vary from 0% to 71%,^{17, 32} with marked differences depending on the geographic location of the patient population. Suzuk *et al*³³ have suggested that the role of HPV may be more pronounced in areas of the world with a high incidence of the disease, and may be less important in areas with moderate or low risk for oesophageal carcinomas. Benamouzig *et al*¹⁷ failed to detect any HPV DNA sequences in patients from two areas of France with oesophageal carcinomas, one area with the highest incidence (28.6 per 100 000 population) and the other with an intermediate incidence (12 per 100 000), by PCR using consensus and type specific primer sets.

Recently, the incidence of oesophageal carcinoma in Japan has gradually increased with an estimated 11.1 per 100 000 population intermediate value.³⁴ Previous studies of Japanese patients with oesophageal carcinomas demonstrated rates of HPV DNA detection from 0% to 33.8% (0 of 34 cases (PCR and Southern blot methods),³⁵ 3 of 45 (PCR),³⁶ 2 of 15 (immunohistochemistry),³⁷ 1 of 4 (PCR and Southern blot),¹⁵ and 24 of 71 cases (ISH)³⁸). In the present study, HPV DNA sequences could not be detected in any oesophageal tumour DNA, globin sequences were generally amplified, and positive controls were always positive. Although one reason for the variation in frequency could be differences in the sensitivity and specificity of the techniques applied, we conclude from the present data that HPV is not of aetiological significance for the majority of oesophageal carcinomas in Japanese patients.

The presence of HPV in adenocarcinomas has been documented in a few reports.^{18, 19}

Cheng *et al*¹⁰ recently demonstrated HPV DNA sequences in colorectal carcinomas, suggesting a possible viral role, especially of type 16, in large bowel cancer. However, Shah *et al*³⁰ cast doubt on this association because all tumour DNA in their study were negative for HPV sequences. To our knowledge, there are no previous studies in which detection of HPV DNA in gastric carcinomas was attempted; we failed to detect HPV genomic sequences in any of a large series of adenocarcinomas.

In conclusion, the present study provided evidence that HPV is not likely to be a major factor in carcinogenesis of either the oesophagus or the stomach in the Japanese population.

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