

Short communication

Physical status of HPV-16 in esophageal squamous cell carcinoma

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Received in revised form 18 April 2004; accepted 20 April 2004

Abstract

Background: Infection with high-risk human papillomavirus (HPV) has been implicated as one of the risk factors of esophageal squamous cell carcinoma (ESCC). Integration of viral DNA into host genome is essential for carcinogenesis since it promotes disruption of the HPV E2 gene, leading to abnormal expression of E6 and E7 oncoproteins. **Objectives and study design:** To investigate the viral integration status of HPV-16 infection in ESCC, 35 HPV-positive ESCC specimens collected from Chinese patients were subject to real-time quantitative PCR for determination of physical status of HPV-16 by analyzing the ratios of E2 to E6 genes. **Results:** Our results showed that only 8.6% (3/35) of the HPV-16 positive specimens harbored exclusively the episomal form (i.e. E2/E6 ratio ≥ 1), whereas the remaining 91.4% contained either only the integrated form (5.7%, with E2/E6 ratio = 0) or a mixture of episomal and integrated forms of viral molecules (85.7%, with E2/E6 ratios > 0 but < 1). Amongst the 30 cancer specimens carrying mixed integrated and episomal forms, 28 had E2/integrated E6 ratios of less than 1, indicating a predominance of integrated form of viral genes in these lesions. **Conclusion:** Our finding of frequent integration of viral DNA in the host genome suggests that integration HPV-16 is common in ESCC from Chinese patients and implies that HPV infection may play a role in the pathogenesis of ESCC.

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Keywords: Esophageal cancer; Esophageal squamous cell carcinoma; HPV; Human papillomavirus; Integration; Physical status

1. Introduction

Human papillomavirus (HPV) has been implicated as one of the risk factors of esophageal squamous cell carcinoma (ESCC) since the observation of condyloma-like lesions in esophageal squamous cell carcinomas some 20 years ago (Syrjanen, 1982). However, unlike cervical carcinoma in which an almost 100% association with HPV is reached, the causal role of HPV infection in the development of ESCC remains controversial partly due to the wide variations in reported infection rates among different studies. To date, the reported HPV infection rates in ESCC, detected as presence

of viral proteins or DNA sequences, vary from 0% to over 60% worldwide (Syrjanen, 2002). Apart from demonstrating the presence of HPV DNA in esophageal lesions so as to establish an etiological link between HPV and esophageal cancer, an important question that should be addressed is whether integration of viral sequences into the host genome, often regarded as a hallmark of HPV-associated cervical carcinogenesis, also occurs in ESCC. In cervical cancer, integration of the high-risk HPV-16 and HPV-18 types into the cell genome occurs early in cancer development, and is correlated with poor prognosis and shortened disease-free survival (Kalantari et al., 1998; Vernon et al., 1997). The role of HPV in malignant transformation in the uterine cervix can be explained by the frequent and preferential disruption of the viral E2 open reading frames (ORF) during integration (Choo et al., 1987). Since E2 is a negative regulator of the E6/E7 promoter, this process leads to increased expression of E6 and E7 viral oncoproteins which target the p53 and

Abbreviations: ESCC, esophageal squamous cell carcinoma; HPV, human papillomavirus; ORF, opening reading frame; PCR, polymerase chain reaction

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pRb tumor suppressor proteins, respectively, to downregulate their anti-tumor functions (Dyson et al., 1989; Scheffner et al., 1990). It has also been suggested that integration of viral sequences close to or within genomic regions encoding cancer-related genes may further contribute oncogenic effects (Couturier et al., 1991; Durst et al., 1987; Reuter et al., 1998). We previously reported detection of relatively low copy numbers of HPV-16 and HPV-18 in ESCC collected from different areas of China using a TaqMan-based 5' exonuclease real-time assay (Si et al., 2003). However, low viral load does not necessarily reflect a lack of viral integration since cervical cancer cells containing the integrated form of HPV-16 are known to have a lower viral copy number than cells harboring episomal HPV DNA (Berumen et al., 1995). In the present study, we used a real-time quantitative PCR assay to determine the physical status of HPV-16 in our specimens by analyzing the ratios of E2 and E6 genes. This highly sensitive approach has been used to demonstrate integration of HPV-16 into the host genome in cervical intraepithelial neoplasia and invasive cancer, (Nagao et al., 2002; Peitsaro et al., 2002) and in squamous cell carcinomas of the head and neck (Koskinen et al., 2003).

2. Materials and methods

Thirty-five ESCC specimens previously determined to be HPV-16 positive by PCR (Si et al., 2003) were examined for physical status of HPV. The collection comprised nine freshly frozen specimens from Hong Kong; 15 frozen specimens from Sichuan Province; one frozen specimen from Linxian County in Henan Province; six paraffin-embedded specimens from Xi'an, Shaanxi Province; and six paraffin-embedded specimens from Shantou, Guangdong Province. In addition, 10 cervical cancer specimens were included to validate the detection system used in the study. The copy numbers of HPV E2 and E6 ORFs were assessed using a TaqMan-based 5' exonuclease quantitative real-time PCR assay based on DNA amplification of a 76 bp sequence of the E2 ORF and a 81 bp sequence of the E6 ORF in the presence of HPV-16 E2- and E6-specific hybridization probes, respectively (Peitsaro et al., 2002). The primers and probe for the E2 assay were designed to recognize the E2 hinge region of the E2 ORF which is most often deleted upon HPV-16 integration in cervical carcinomas (Kalantari et al., 1998; Vernon et al., 1997). For each specimen, identical amounts of DNA were quantified for the E6 and E2 sequence of HPV-16. Each specimen was assayed three times. The PCR amplification was performed in a 25 μ l volume containing 1 \times platinum quantitative PCR SuperMix (Life Technologies, Gaithersburg, MD, USA) with 4 mM MgCl₂, 300 nM E2 or E6 specific primers, 100 nM dual-labeled (5'FAM and 3'TAMRA) E2 or E6 fluorogenic hybridization probe, and 1–2 μ l of DNA template. A standard curve generated using serially diluted purified HPV-16 plasmid DNA (kindly provided

by Dr. H. zur Hausen, Deutsches Krebsforschungszentrum, Heidelberg, Germany) was included in each experiment. The amplification ramp included two hold programs of 2 min at 50 °C and 10 min at 95 °C, followed by a two-step PCR cycle with a melting step for 15 s at 95 °C and an annealing step for 1 min at 60 °C, for a total of 45 cycles. The threshold cycle (Ct) was determined by the built-in software, with the baseline set automatically to 10 standard deviations above background in the first 10–15 cycles.

The ratio of E2 to E6 copy numbers were calculated to determine the physical status of HPV-16 viral gene. HPV-16 in pure episomal form was expected to have equivalent copy numbers of E2 and E6 genes (i.e. E2/E6 ratio = 1), whereas preferential disruption of E2 upon viral integration should result in less E2 gene copies than E6. This means that an E2/E6 ratio of less than 1 would indicate the presence of both integrated and episomal forms, while a ratio of 0 would indicate the presence of integrated form only. The copy number of integrated E6 was calculated by subtracting the copy number of E2 (episomal) from the total copy number of E6 (episomal and integrated). The ratio of E2 to integrated E6 represents the amount of the episomal form in relation to the integrated form.

Values less than 1 indicate predominance of the integrated form. DNA extracted from cervical carcinoma cell line SiHa, known to harbor pure integrated form of HPV-16 gene with disruption of the E2 and E4 ORFs (Baker et al., 1987; el Awady et al., 1987), was used as control for E2 (negative) and E6 (positive) amplification.

3. Results and discussion

Integration of viral sequences into the host genome seems to play an important role in progression to malignancy in human cancers with a viral etiology (Feitelson, 1999; Tonon et al., 2001). In this study, we determined the physical status of HPV-16 in ESCC using quantitative real-time PCR.

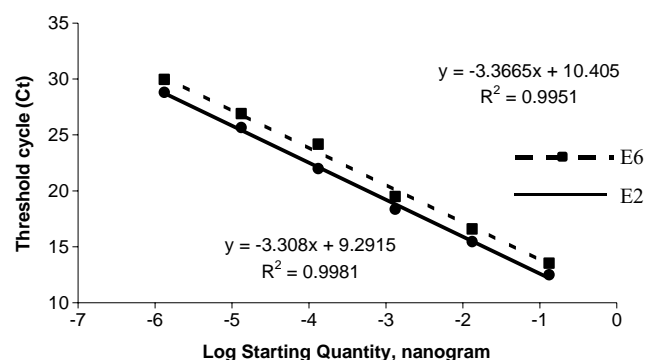


Fig. 1. Comparison of PCR amplification efficiencies for HPV-16 E6 and E2. A 6-point 10-fold dilution series of HPV-16 plasmid DNA (1 \times 10⁻⁶ to 1 \times 10⁻¹ ng) was amplified for both HPV-16 E6 and E2. The PCR amplification efficiencies were found to be very similar for the two reactions.

Compared to other methods such as Southern blotting, the method has the advantages of being highly sensitive and requires no post-PCR analysis so that even very low copy numbers of integrated HPV DNA can be detected in the presence of excess episomal DNA (Peitsaro et al., 2002). In addition, the TaqMan-based detection system used in this study included the use of the dUTP protocol, which minimizes carry-over contamination (Longo et al., 1990). Since the results of this study were based on the calculation of the

relative ratio between two parameters (i.e. E2 and E6 copy numbers) determined in two separate assays, we also validated the assay by comparing the amplification efficiencies of the real-time amplification systems for E2 and E6 ORFs using serially diluted HPV-16 plasmid DNA. We found that the two assays had very similar amplification efficiencies, as reflected by the nearly identical slopes of the amplification curves (Fig. 1). This validation also allays the concern that formalin-fixation and paraffin embedding of some of

Table 1
HPV-16 viral load and physical status in esophageal squamous cell carcinoma and cervical carcinoma specimens

Origin	Case no.	HPV-16 copies/cell	E2/E6 ratio	E2/integrated E6 ratio	Physical status (HPV-16)
Esophageal cancer					
Hong Kong	HKFT19	0.6	0.22	0.29	Mixed
	HKFT30	1.5	0.08	0.09	Mixed
	HKFT35	1.1	0.32	0.47	Mixed
	HKFT50	0.7	0.41	0.71	Mixed
	HKFT52	0.1	0.43	0.75	Mixed
	HKFT58	3.6	0.15	0.18	Mixed
	HKFT70	1.3	0.16	0.19	Mixed
	HKFT77	0.3	0.07	0.07	Mixed
	HKFT91	5.6	0.00	0.00	Integrated
Sichuan	SCFT2	14.3	0.23	0.3	Mixed
	SCFT18	6.6	0.44	0.78	Mixed
	SCFT19	0.02	0.59	1.44	Mixed
	SCFT27	9.1	0.65	1.88	Mixed
	SCFT29	57.2	0.44	0.79	Mixed
	SCFT35	28.3	0.44	0.77	Mixed
	SCFT36	5.6	4×10^{-6}	0.04	Mixed
	SCFT45	7.3	0.23	0.3	Mixed
	SCFT49	4.1	0.27	0.38	Mixed
	SCFT83	156.9	1.08	No integration	Episomal
	SCFT88	118.7	0.45	0.81	Mixed
	SCFT93	2.1	0.22	0.29	Mixed
	SCFT100	137.7	1.57	No integration	Episomal
	SCFT104	81.4	0.1	0.11	Mixed
SCFT119	43.1	0.16	0.19	Mixed	
Linxian	LXFT29	21.4	0.16	0.19	Mixed
Xi'an	XAPT1	0.0004	0.40	0.65	Mixed
	XAPT4	1.6	0.02	0.02	Mixed
	XAPT14	157.2	0.02	0.02	Mixed
	XAPT46	0.004	0.08	0.09	Mixed
	XAPT50	0.0002	0.10	0.11	Mixed
	XAPT56	0.004	0.18	0.23	Mixed
Shantou	STPT10	12.0	1.27	No integration	Episomal
	STPT11	24.0	0.22	0.28	Mixed
	STPT12	17.1	0.08	0.08	Mixed
	STPT18	7×10^{-6}	0.00	0.00	Integrated
Cervical cancer					
	CC1	255.4	0.00	0.00	Integrated
	CC2	114.2	0.70	2.238	Mixed
	CC3	255	0.25	0.333	Mixed
	CC4	838	0.25	0.340	Mixed
	CC5	189.2	0.30	0.419	Mixed
	CC6	91.4	0.21	0.259	Mixed
	CC7	869.4	0.07	0.073	Mixed
	CC8	5.2	6.748	-1.174	Episomal
	CC9	527.6	0.00	0.00	Integrated
	CC10	364.2	0.107	0.119	Mixed
SiHa cell line		3.7	0.00	0.00	Integrated

the specimens might affect the amplification efficiency of the PCR assays (Si et al., 2003) since both assays would be affected to a similar extent, thus preserving the accuracy of the E2/E6 ratio. The validity of the assay was further confirmed by the accurate determination of the physical status of HPV-16 in SiHa cervical carcinoma cell line, which harbors pure integrated form of HPV-16 gene. In all the experiments, SiHa DNA consistently showed a lack of E2 amplification but was positive for E6 amplification (Table 1).

Our data showed HPV-16 integration in 91.4% (32/35) of the HPV-positive ESCC specimens (Table 1). These include 2 (5.7%) specimens without detectable E2 sequence, indicating complete integration of viral genes into the host genome, and 30 (85.7%) specimens with E2/E6 ratios between 0 and 1, which were indicative of presence of mixed integrated and episomal forms. Amongst the 30 ESCC specimens harboring mixed forms of viral molecules, 28 had E2/integrated E6 ratios of less than 1, suggesting a predominance of the integrated form of viral genes. The pure episomal form of HPV-16 DNA (i.e. E2/E6 ratio ≥ 1) was detected in only 3/35 (8.6%) of our ESCC specimens. Although our HPV-16 positive sample size was too small to allow us to determine if there was any difference in HPV-16 physical status among the different geographic areas, it was clear that the majority of cases from each area contained a mixture of episomal and integrated viral molecules. We conclude that HPV-16 integration is a common phenomenon in HPV-positive ESCC cancer patients. This is similar to the situation in cervical cancers, in which viral integration can be detected in as high as 63–100% of the cases (90% in this study) and is frequently detected together with the episomal form (Badaracco et al., 2002; Kristiansen et al., 1994; Park et al., 1997; Tonon et al., 2001; Vernon et al., 1997). As in the esophageal samples, the E2/integrated E6 ratios indicated that the integrated form of viral genes was predominant in the cervical cancer specimens harboring the mixed form of HPV-16. The viral copy numbers in cervical cancer ranged from 5.2 to 869.4, with a mean value of 351 copies per genome equivalent (Table 1).

Integration of HPV not only leads to deletion of the E2 gene and deregulated expression of E6 and E7 in cervical carcinoma (Choo et al., 1987), it has also been shown to impart selective growth advantage in cervical epithelial cells in vitro (Jeon et al., 1995). Conversely, exogenous expression of HPV-16 E2 gene results in cellular growth arrest and senescence in cervical cancer cell lines through repression of E6/E7 promoter (Francis et al., 2000; Hwang et al., 1993). Thus, determination of the physical status of HPV DNA is important for understanding of the role of HPV infection in the development of ESCC. The physical status of HPV has rarely been reported in esophageal cancer. Based on interpretation of non-isotopic in situ hybridization staining patterns, Cooper et al. found that ESCC samples from South Africa showed a punctuate staining pattern characteristic of integrated HPV DNA (Cooper et al., 1995). Like South Africa, China has exceptionally high incidence of esophageal cancer, and an earlier Southern hybridization

study provided some indication of HPV viral integration in ESCC from Linxian (Chang et al., 1992). Linxian is a county in the Henan Province of China renowned for having the highest incidence and mortality rate of esophageal cancer in the world. However, the etiology of esophageal cancer in this geographic area is mainly linked to dietary factors (Yang, 2000), and we have also reported a very low HPV infection rate (2%) in ESCC specimens from this area (Si et al., 2003). Nevertheless, determination of viral physical status in this study showed the presence of integrated HPV-16 DNA together with episomal form in the only HPV-16 positive specimen from Linxian.

It has been suggested that heavy viral loads increase the probability of integration into chromosomal sites since progression of cervical intraepithelial neoplasia lesions was found to be associated with heavy loads of integrated HPV (Peitsaro et al., 2002). We were the first to report the viral loads of HPV-16 in ESCC from China (Si et al., 2003). Using a real-time quantitative PCR assay based on the amplification of a 180 bp fragment from the 3' part of the E1 open reading frame with HPV-16 type-specific probe, we found that the HPV-16 copy numbers in the esophageal specimens were in the range of ≤ 1 –157 copies per genome equivalent (Table 1) (Si et al., 2003). The relatively low viral load (≤ 10 copies per genome equivalent) in the majority of positive tumors would seem to suggest a lower chance of viral interaction with the host genome. However, viral integration is likely to occur early in cancer development, as indicated by episomal amplification being often detected in cervical precancerous lesions, and the predominance of integrated forms in invasive cancer (Badaracco et al., 2002; Tonon et al., 2001). Considering the presence of relatively low viral load of HPV DNA in some cervical carcinoma cell lines, e.g. SiHa (1–2 copies of HPV-16 per cell) and HeLa (10–50 copies of HPV-18 per cell), it is possible that the low viral load in some esophageal tumor cells, particularly those with integrated viral genome, is sufficient to lead to or promote carcinogenesis, especially if combined with other genetic changes.

In cervical cancer, it has been shown that cells with diffuse staining signals (by non-isotopic in situ hybridization) representing viral episomes have a higher HPV-16 load than cells with the punctuate/integrated pattern (Berumen et al., 1995). It was therefore interesting to note that two of the three ESCC specimens carrying pure episomal form of HPV-16 DNA had relatively high viral loads (138 and 157 copies, respectively) compared to the samples containing integrated viral sequences. Conversely, one of the two samples with exclusively integrated form of HPV-16 had the lowest viral load. However, we did not find any significant correlation between viral load and E2/E6 ratio (Spearman rank correlation Coefficient (ρ) = 0.272, $P > 0.1$). The lack of correlation may be due to non-uniform distribution of HPV-infected cells in each specimen and the averaging of viral loads of both HPV-positive and -negative cells within the sample during assay. It could also be that the physical

status of the virus differed from cell to cell. However, our finding of frequent integration of viral DNA in ESCC of Chinese patients strongly suggests that HPV infection may be one of the multiple risk factors of ESCC. Further work is needed to elucidate the underlying molecular mechanisms and the genetic changes associated with HPV infection in esophageal cancer.

Acknowledgements

This work was supported by a grant from the Research Grants Council of the Hong Kong Special Administrative Region, China (Project No. HKU 7057/99M). We thank Prof. Alfred K.Y. Lam (Pathology, School of Medicine, James Cook University, Australia), Prof. Z.Y. Shen (Shantou Medical University, PR China) and Prof. L.D. Wang (Laboratory for Cancer Research, Henan Medical University, PR China) for providing some of the samples used in this study. We also thank Ms. Alla Li for excellent technical assistance.

References

- Badaracco G, Venuti A, Sedati A, Marcante ML. HPV-16 and HPV-18 in genital tumors: significantly different levels of viral integration and correlation to tumor invasiveness. *J Med Virol* 2002;67:574–82.
- Baker CC, Phelps WC, Lindgren V, Braun MJ, Gonda MA, Howley PM. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. *J Virol* 1987;61:962–71.
- Berumen J, Unger ER, Casas L, Figueroa P. Amplification of human papillomavirus types 16 and 18 in invasive cervical cancer. *Hum Pathol* 1995;26:676–81.
- Chang F, Syrjanen S, Shen Q, Wang L, Wang D, Syrjanen K. Human papillomavirus involvement in esophageal precancerous lesions and squamous cell carcinomas as evidenced by microscopy and different DNA techniques. *Scand J Gastroenterol* 1992;27:553–63.
- Choo KB, Pan CC, Han SH. Integration of human papillomavirus type 16 into cellular DNA of cervical carcinoma: preferential deletion of the E2 gene and invariable retention of the long control region and the E6/E7 open reading frames. *Virology* 1987;161:259–61.
- Cooper K, Taylor L, Govind S. Human papillomavirus DNA in oesophageal carcinomas in South Africa. *J Pathol* 1995;175:273–7.
- Couturier J, Sastre-Garau X, Schneider-Maunoury S, Labib A, Orth G. Integration of papillomavirus DNA near myc genes in genital carcinomas and its consequences for proto-oncogene expression. *J Virol* 1991;65:4534–8.
- Durst M, Croce CM, Gissmann L, Schwarz E, Huebner K. Papillomavirus sequences integrate near cellular oncogenes in some cervical carcinomas. *Proc Natl Acad Sci USA* 1987;84:1070–4.
- Dyson N, Howley PM, Munger K, Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 1989;243:934–7.
- el Awady MK, Kaplan JB, O'Brien SJ, Burk RD. Molecular analysis of integrated human papillomavirus 16 sequences in the cervical cancer cell line SiHa. *Virology* 1987;159:389–98.
- Feitelson MA. Hepatitis B virus in hepatocarcinogenesis. *J Cell Physiol* 1999;181:188–202.
- Francis DA, Schmid SI, Howley PM. Repression of the integrated papillomavirus E6/E7 promoter is required for growth suppression of cervical cancer cells. *J Virol* 2000;74:2679–86.
- Hwang ES, Riese DJ, Settleman J, Nilson LA, Honig J, Flynn S, Di-Maio D. Inhibition of cervical carcinoma cell line proliferation by the introduction of a bovine papillomavirus regulatory gene. *J Virol* 1993;67:3720–9.
- Jeon S, Allen-Hoffmann BL, Lambert PF. Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. *J Virol* 1995;69:2989–97.
- Kalantari M, Karlsen F, Kristensen G, Holm R, Hagmar B, Johansson B. Disruption of the E1 and E2 reading frames of HPV 16 in cervical carcinoma is associated with poor prognosis. *Int J Gynecol Pathol* 1998;17:146–53.
- Koskinen WJ, Chen RW, Leivo I, Makitie A, Back L, Kontio R, Suuronen R, Lindqvist C, Auvinen E, Molijn A, Quint WG, Vaheri A, Aaltonen LM. Prevalence and physical status of human papillomavirus in squamous cell carcinomas of the head and neck. *Int J Cancer* 2003;107:401–6.
- Kristiansen E, Jenkins A, Holm R. Coexistence of episomal and integrated HPV16 DNA in squamous cell carcinoma of the cervix. *J Clin Pathol* 1994;47:253–6.
- Longo MC, Berninger MS, Hartley JL. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. *Gene* 1990;93:125–8.
- Nagao S, Yoshinouchi M, Miyagi Y, Hongo A, Kodama J, Itoh S, Kudo T. Rapid and sensitive detection of physical status of human papillomavirus type 16 DNA by quantitative real-time PCR. *J Clin Microbiol* 2002;40:863–7.
- Park JS, Hwang ES, Park SN, Ahn HK, Um SJ, Kim CJ, Kim SJ, Namkoong SE. Physical status and expression of HPV genes in cervical cancers. *Gynecol Oncol* 1997;65:121–9.
- Peitsaro P, Johansson B, Syrjanen S. Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. *J Clin Microbiol* 2002;40:886–91.
- Reuter S, Bartelmann M, Vogt M, Geisen C, Napierski I, Kahn T, Delius H, Lichter P, Weitz S, Korn B, Schwarz E. APM-1, a novel human gene, identified by aberrant co-transcription with papillomavirus oncogenes in a cervical carcinoma cell line, encodes a BTB/POZ-zinc finger protein with growth inhibitory activity. *EMBO J* 1998;17:215–22.
- Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 1990;63:1129–36.
- Si HX, Tsao SW, Poon CS, Wang LD, Wong YC, Cheung AL. Viral load of HPV in esophageal squamous cell carcinoma. *Int J Cancer* 2003;103:496–500.
- Syrjanen KJ. Histological changes identical to those of condylomatous lesions found in esophageal squamous cell carcinomas. *Arch Geschwulstforsch* 1982;52:283–92.
- Syrjanen KJ. HPV infections and oesophageal cancer. *J Clin Pathol* 2002;55:721–8.
- Tonon SA, Picconi MA, Bos PD, Zinovich JB, Galuppo J, Alonio LV, Teyssie AR. Physical status of the E2 human papilloma virus 16 viral gene in cervical preneoplastic and neoplastic lesions. *J Clin Virol* 2001;21:129–34.
- Vernon SD, Unger ER, Miller DL, Lee DR, Reeves WC. Association of human papillomavirus type 16 integration in the E2 gene with poor disease-free survival from cervical cancer. *Int J Cancer* 1997;74:50–6.
- Yang CS. Vitamin nutrition and gastroesophageal cancer. *J Nutr* 2000;130:338S–9S.