Low Prevalence of Human Papillomavirus Infection in Esophageal Squamous Cell Carcinomas From North America:

Analysis by a Highly Sensitive and Specific Polymerase Chain Reaction–Based Approach

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Several studies have documented the frequent occurrence of human papillomavirus (HPV) DNA in esophageal squamous cell carcinomas (ESCC) in patients from geographic regions where the incidence of this type of cancer is high, such as parts of China. However, the prevalence of HPV infection in ESCC in patients from low incidence geographic regions, such as North America, remains controversial. Therefore, this study evaluates the prevalence of HPV in ESCC in patients from North America, a region where the population is considered at low risk for the development of this neoplasm. ESCCs in 51 patients from three North American cities were analyzed for the presence of HPV DNA by a highly sensitive and specific polymerase chain reaction (PCR) method. Tumor DNA was extracted from formalin-fixed, paraffin-embedded tissue specimens and assayed by PCR using an L1 HPV consensus sequence primer, as well as HPV 16 and HPV 18 E7 region primers. The use of consensus primers to the L1 region allows for detection of most known HPV types and many novel HPV types. Appropriately sized reaction products were analyzed by restriction fragment length polymorphism (RFLP) to confirm the presence and type of HPV, and to exclude products produced by amplification of human DNA sequences. After complete analysis, only one case (2%) of ESCC was HPV DNA positive. This case was independently confirmed using L1 and E7 consensus primers as HPV type 16 and was the only case that tested positive with either assay. These results show that, in contrast to geographic regions where ESCC is prevalent, HPV infection occurs infrequently in association with ESCC in patients from North America. HUM PATHOL 28:174–178. Copyright © 1997 by W.B. Saunders Company

Key words: **human papillomavirus, esophagus, squamous cell carcinoma, polymerase chain reaction.**

Abbreviations: **HPV, human papilloma virus; ESCC, esophageal squamous cell carcinoma; AJCC, American Joint Committee on Cancer; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; dCTP, deoxycytidine triphosphate.**

Squamous cell carcinoma is the most common malignant epithelial neoplasm of the esophagus worldwide.[1-4] However, the risk of acquiring esophageal squamous cell carcinoma (ESCC) varies widely among different geographic regions. The annual incidence of ESCC in North America and other low-risk regions ranges from 1.1 to 2.6 per 100,000,[1,4,5] whereas the annual incidence in high-risk regions, such as Northern China and regions of Iran exceeds 130 per 100,000.[2,3] Moreover, within some high risk areas, striking differences in the incidence of ESCC occur over short distances.[3] For example, in the Henan province of China, the highest mortality rate of 161 per 100,000 (for men) occurs in Linxian, whereas the mortality rate for men in Fanxian, approximately 100 miles to the north, is only 27 per 100,000.[3]

The wide geographic variation in incidence of ESCC within genetically homogeneous populations implies a causal role for environmental factors in the pathogenesis of ESCC. Epidemiological studies have shown that environmental factors, including tobacco, alcohol, and certain dietary products, are associated with the development of ESCC in both high and low-risk geographic regions.[6-12]

Human papillomavirus (HPV) infection has been described in a high proportion of ESCCs in high-risk regions worldwide. For example, HPV-like DNA sequences were detected in 24% to 60% of ESCCs from high-risk regions, such as parts of China, South Africa, and Iran.[13-16] These data have led to the hypothesis that, similar to cervical squamous cell carcinoma, HPV infection is associated with the development of ESCC.[17]

In contrast to geographic regions at high-risk for ESCC, only a few cases of ESCC in patients from low-risk regions, such as North America, have been systematically analyzed for HPV DNA.[16,18,19] Therefore, this study evaluates the prevalence of HPV DNA in a cohort of North American patients with ESCC using a highly sensitive and specific polymerase chain reaction (PCR) technique.[20,21] This method increases the sensitivity over traditional PCR-based approaches and allows for the simultaneous identification of the specific HPV type in addition to nonspecific amplified non-HPV DNA sequence contaminants.[20,21] The use of consensus primers for distinct regions of the HPV genome detects most known HPV types, including types 6, 11, 16, 18, 31, and 33, and can also detect novel HPV types based on RFLP digestion patterns.[20-24] We used this method to evaluate

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the prevalence of HPV infection in 51 cases of ESCC from North American patients.

METHODS

Study Group

The study group consisted of 51 patients who had undergone esophagectomy for resection of primary ESCC. These cases were retrieved from the surgical pathology files of Mt. Sinai Hospital (Toronto, Canada); Brigham and Women's Hospital (Boston, MA); New England Deaconess Hospital (Boston, MA); and Baptist Memorial Hospital (Memphis, TN), with all three cities being equally represented. Only patients of North American descent were selected, and those who either immigrated to North America or had extensive travel histories were excluded from this study.

All resection specimens were fixed in 10% buffered-formalin, routinely processed, embedded in paraffin, and stained with hematoxylin-eosin. The following parameters were evaluated: anatomic site (upper, middle, or lower esophagus); histological grade (well, moderately, or poorly differentiated); depth of invasion; and lymph node metastases. Information regarding the anatomic site was not available in 13 cases. The pathological American Joint Committee on Cancer (AJCC) stage was determined for each case. A representative paraffin block from each case containing invasive squamous cell carcinoma was selected for DNA isolation.

TABLE 1. Clinicopathologic Features of Patients with Esophageal Squamous Cell Carcinoma

Clinicopathologic Feature	Result
Mean patient age (yr)	67 (range, 44 to 84)
Male-female Ratio	32/19 (1.7:1)
Mean tumor size (cm)	3.9 (range, 0.5 to 11)
Tumor site, n (%)	
Proximal esophagus	3 (6)
Middle esophagus	13 (25)
Distal esophagus	22 (43)
Not determined	13 (25)
Histological grade, n (%)	
Well differentiated	3 (6)
Moderately differentiated	40 (78)
Poorly differentiated	8 (16)
Depth of invasion, n (%)	
Mucosa	1 (2)
Submucosa	3 (6)
Muscularis propria	15 (29)
Adventitia	32 (63)
Patients with lymph node metastases, n (%)	17 (33)
AJCC stage, n (%)	
I	4 (8)
IIA	20 (39)
IIB	8 (16)
III	12 (23)
IV	7 (14)

Archival DNA Isolation

Serial tissue sections (6 μm thick) were cut from each paraffin block using disposable microtome blades. Routine precautions to avoid cross-contamination were undertaken as previously described.[21] Four tissue sections were placed in an Eppendorf microcentrifuge tube with 500 μL of proteinase digestion buffer, 50 mmol/L Tris (hydroxymethyl) aminomethane, pH 8.5, 1 mmol/L ethylenediaminetra-acetic acid, 200 μg/mL proteinase K, and 0.5% Tween 20 (Sigma Chemical, St. Louis, MO). After incubation for 4 to 8 hours at 62°C, 250 μL of a 50% slurry of Chelex (Bio-Rad, Hercules, CA) in distilled water were added and the mixtures boiled for 10 minutes. After centrifugation for 5 minutes at 14,000 revolutions per minute, the paraffin surface was pierced, and the aqueous phase removed and stored frozen at −20°C.

HPV DNA Analysis

For PCR analysis, HPV L1 region consensus primers (MY9: 5'CGT CCM ARR GGA WAC TGA TC-3'; MY11: 5'-GCM CAG GGW CAT AAY AAT GG-3'),[21,23,24] or a cocktail of HPV 16 E7 region primers (5'-TGT GTC CTG AAG AAA AGC AAA GAC-3' and 5'-AGC TGT CAT TTA ATT GCT CAT AAC AGT A-3'), and HPV 18 E7 region primers (5'-CGA ACC ACA ACG TCA CAC AAT-3' and 5'-GCT TAC TGC TGG GAT GCA CA-3')[20] were used. The latter primers also detect HPV 33. PCR using β-globin gene primers (5'-GGA AAA TAG ACC AAT AGG CAG-3' and 5'-GGT TGG CCA ATC TAC TCC CAG G-3') was used as a control for DNA adequacy. To control for archival DNA quality, only cases with amplifiable β-globin DNA were included in this study. PCR analysis was performed according to the following protocol.[20-22,25,26] One microliter of DNA sample was amplified in a 25-μL reaction mixture with 0.5 μmol/L primers for 35 cycles in a thermocycler (Perkin-Elmer, Norwalk, CT). Denaturing, annealing, and extension times and temperatures were 30 seconds at 95°C, 30 seconds at 55°C for L1 primers or 53°C for E7 primers, and 1 minute at 72°C, respectively. DNA products were resolved on 2% agarose gels and detected by ethidium bromide staining. Appropriately sized PCR products (450 base pairs with L1 primers, 224 base pairs with HPV 16 E7 primers, and 159 base pairs with HPV 18 E7 primers) were then analyzed by restriction fragment length polymorphism (RFLP).[27]

Restriction Fragment Length Polymorphism Analysis

For RFLP analysis, samples were amplified under the conditions described previously but with the addition of 2 μCi of ^{32}P-deoxycytidine triphosphate (dCTP). This enhances sensitivity and facilitates analysis on polyacrylamide gels.[20,21] For the L1 region primer reactions, 10 μL of PCR product was digested in a 25-μL reaction containing 10 units each of *Pst* I, *Rsa* I, and *Hae* III.[25] For the E7 region primers, 10 μL of product were digested with 10 units of *Alu* I. Digestion products were separated on 6% polyacrylamide gels and detected by autoradiography for 12 hours to 26 hours. Digestion products were sized and compared with HPV 6, 16, and 18 positive controls digested under similar conditions. Determination of the specific HPV type was based on comparison with known HPV standards and established restriction maps of the amplified regions.[25] This method can identify most known HPV types, such as types 6, 11, 16, 18, 31, and 33.[22,24,25] Novel restriction patterns corresponding to as yet uncharacterized HPV types may also be detected with this method.[22,24,25] However, novel HPV types were not identified in this study (see Results).

RESULTS

The clinical and pathological features of the patients and their tumors are summarized in Table 1. DNA isolated from each tumor was amplified with L1 region consensus primers and the PCR products were

analyzed on ethidium bromide-stained agarose gels (Fig 1A). An approximately 450–base pair PCR product, the size predicted for amplification of HPV DNA with these primers, was identified in 19 of 51 ESCC. To determine whether these PCR products were derived from amplification of HPV or non-HPV (ie, human DNA), RFLP analysis of the ^{32}P-labeled PCR products was performed. After RFLP analysis, only 1 ESCC produced an RFLP pattern consistent with amplified HPV DNA sequences (Fig 2A). Thus, amplification of tumor DNA with L1 region consensus primers detected HPV DNA in only 1 of 51 ESCC (2%). The restriction fragment sizes (217, 68, and 66 base pairs) corresponded to HPV type 16 (Fig 2A). Despite the fact that many HPV types can be detected by these primers, no cases showed a restriction pattern that corresponded to other HPV types. A nonspecific band identical to that detected in control human DNA was present in most samples (Fig 2A).

To confirm the 1 HPV-positive ESCC, and to enhance the sensitivity of our analysis, we used a similar PCR- and RFLP-based assay using primers for the E7 region of HPV 16 and HPV 18. Analysis of the PCR products generated by amplification of ESCC DNA with E7 primers on ethidium bromide–stained gels (Fig 1B) resulted in detectable products near or identical to an HPV-16 (224–base pair) band in 8 of 51 cases. To determine if these PCR products were derived from HPV DNA, we analyzed the ^{32}P-labeled PCR products by RFLP (Fig 2B). The RFLP analysis showed HPV DNA sequences in 1 case. When compared with HPV 16 and 18 standards, the sizes of the fragments generated (121 and 100 base pairs) corresponded to HPV 16 (Fig 2B). This case identified with the E7 primers was the same one that was determined to contain HPV DNA using the L1 primers. Therefore, the two methods independently detected HPV 16 DNA in the same ESCC.

The case that contained HPV DNA was that of a 48-year-old man with an AJCC stage III midesophageal squamous cell carcinoma. The tumor was 5 cm in greatest dimension, moderately differentiated, involved the periesophageal soft tissues, and was metastatic to regional lymph nodes. There was no evidence of coexisting HPV cytopathic effect.

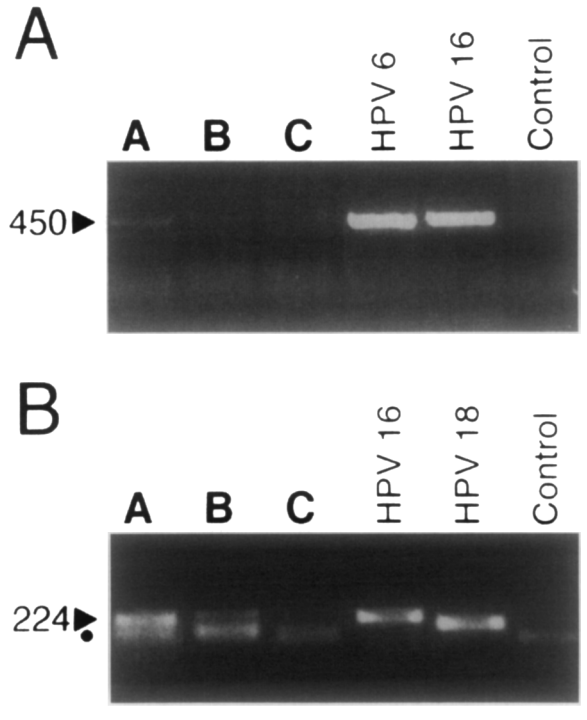

FIGURE 1. Ethidium bromide-stained agarose gels of PCR-amplified ESCC DNA. (A) Amplification using HPV L1 primers. Samples of ESCC tumor DNA were PCR amplified using HPV L1 consensus primers and the resulting products separated on agarose gels. Lane A shows the one positive case identified in this study. A faint band at 450 base pairs is present in lane B, but this sample did not contain HPV DNA when analyzed by RFLP (Fig 2A). The ESCC analyzed in lane C did not contain an identifiable amplified band. (B) Amplification using HPV E7 primers. Samples of ESCC tumor DNA were PCR amplified using HPV E7 primers and the resulting products separated on agarose gels. Lane A shows the one positive case identified in this study. A band at 224 base pairs is present in lane B, but this sample did not contain HPV DNA when analyzed by RFLP (Fig 2B). The ESCC analyzed in lane C did not contain an identifiable amplified band.

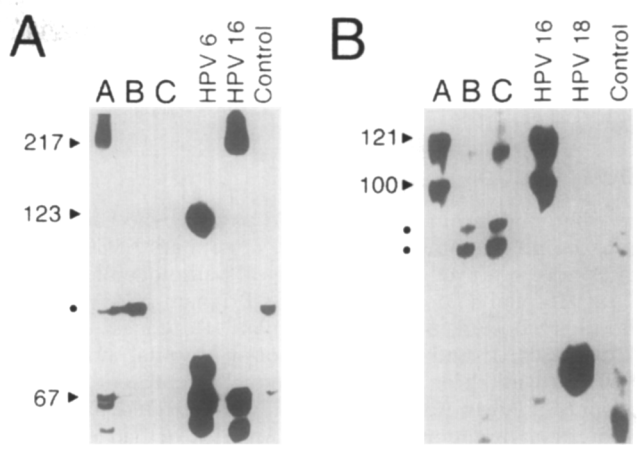

FIGURE 2. RFLP analysis of PCR products after amplification of ESCC DNA. (A) Analysis of products generated with HPV L1 primers. Appropriately sized PCR products generated by amplification of ESCC with L1 consensus primers (450 base pair products) (Fig 1A) were labeled with ^{32}P and digested with *Rsa* I, *Pst* I, and *Hae* III. HPV 6 DNA, HPV 16 DNA, and negative control human DNA were also analyzed. The digestion fragments were separated by polyacrylamide gel electrophoresis. Lane A shows the one positive case identified in this study. Comparison with expected fragment sizes and the HPV 16 control identified the HPV DNA as type 16. Lanes B and C illustrate cases in which ~450 base pair PCR products were detected on ethidium bromide–stained gels. However, the fragments generated on digestion of these PCR products are also present after amplification of human DNA (compare with control). (B) Analysis of products generated with HPV E7 primers. Appropriately sized PCR products generated by amplification of ESCC with E7 primers (224–base pair products) (Fig 1B) were labeled with ^{32}P and digested with *Alu* I. HPV 16 DNA, HPV 18 DNA, and negative control human DNA were also analyzed. The digestion fragments were separated by polyacrylamide gel electrophoresis. Lane A shows the one positive case identified in this study. Comparison with expected fragment sizes and the HPV 16 control identified the HPV DNA as type 16. Lanes B and C illustrate cases in which 224–base pair PCR products were detected on ethidium bromide–stained gels. The case shown in lane B is the same case shown in lane B of Fig 1B. The samples analyzed in lanes B and C do not contain identifiable HPV DNA.

DISCUSSION

Human papillomavirus DNA sequences have been detected by PCR in 24% to 60% of ESCC.[13-16] However, most of these studies evaluated neoplasms of patients from regions considered at high risk for the development of ESCC.[13-16,19,28] Because different etiologic factors may be associated with the development of ESCC in high versus low-risk regions,[3] we undertook this study to evaluate the association of HPV with ESCC from North America, a region considered at low risk for the development of this neoplasm.

In this study, a highly sensitive and specific PCR-based method was used to detect HPV DNA sequences in ESCC from 51 North American patients. Neoplasms were accrued from three North American cities to obtain a broad population sample representative of low-risk regions for ESCC. Our analysis showed the presence of HPV type 16 DNA in only 1 of 51 (2%) ESCC. This positive case was identified by two sets of PCR primers against distinct regions of the HPV genome and verifies the previously described high degree of sensitivity of these parallel PCR assays.[20,21] Our use of consensus primers that detect known, as well as previously unidentified, HPV types[22,24] makes it highly unlikely that we have failed to detect HPV DNA of any type in our cohort of cases. However, one cannot entirely exclude the possibility of false negatives in any study of this kind and that HPV types not specifically tested for may play a role in ESCC.

Because PCR assays are prone to false-positive results, in part because of amplification of human nonviral sequences,[21,29] we demanded confirmation of all appropriately sized PCR products by RFLP analysis. The sequence dependence of the restriction enzymes used in RFLP analysis results in a nucleotide sequence-dependent means of verifying or refuting the viral origin of the amplified DNA.[20,21,29] Because the nonspecific non-HPV bands seen on ethidium bromide–stained gels are lost after digestion with restriction enzymes, they do not complicate such analysis.[21,29] In fact, previous studies by our group, as well as those by others have shown that, by direct sequencing, the nonspecific bands are derived from human DNA.[21,29]

In the one HPV-positive ESCC in this study, RFLP analysis of the PCR products amplified by the L1 and E7 region primers identified the DNA as HPV type 16. Although direct sequencing of viral DNA is considered a preferable method to confirm the presence and type of HPV DNA, the small quantity of amplified DNA present in our case rendered direct sequencing an unsuitable approach. Furthermore, RFLP analysis represented a reliable alternative,[21,25,30] particularly with the use of ^{32}P-dCTP, which affords an enhancement in sensitivity over ethidium bromide-staining.[21] An alternative method used by others to verify the sequence of PCR products was Southern blot analysis.[14] However, one drawback of this technique was that hybridization under low stringency conditions may not eliminate false positives, whereas hybridization under high stringency conditions may fail to confirm positive cases.

Our study represents the first large analysis of HPV in ESCC in patients exclusively from North America. However, several other studies have evaluated ESCC in patient populations from North America in combination with other high, moderate, and low incidence regions. Togawa et al[16] recently analyzed 72 ESCC from high, moderate, and low risk geographic regions (Iran, South Africa, Italy, France, Japan, and the United States) and identified HPV DNA in 17 of 72 (24%) of cases overall including HPV 16 DNA in 2 of 15 (13%) of the cases from the United States. Their study used the same L1 region primers as in our study. However, they performed nested radiolabeled PCR using the first PCR product and a degenerate sense primer internal to the HPV L1 sense primer. It is possible that in an effort to enhance sensitivity, the use of nested primers may have decreased the specificity of the test, or resulted in amplification of human DNA sequences.[29] Alternatively, differences in patient selection may be responsible for the difference in the HPV-positivity rate obtained in our study compared with that of Togawa et al.[16] Our study consisted of an exclusively North American nonimmigrant population. However, in the study by Togawa et al,[16] patients were designated as from the United States if their surgical resection was performed in that country. Therefore, it is possible that some of the patients may not have been truly of North American descent.

In a recent study published in abstract form, Noffsinger et al[31] reported HPV positivity in 1 of 26 (3.8%) ESCC in patients from North America and in 2 of 70 (2.8%) ESCC cases from a low incidence region of China. Kiyabu et al[19] used PCR with E6 region primers to study 13 ESCC in patients from Southern California. They detected HPV DNA in 0 of 13 (0%) cases of ESCC, but in 44 of 75 (59%) squamous cell carcinomas from other anatomic sites including the oropharynx, anus, and vulva. Similarly, in a non–PCR-based Southern blot analysis of nonamplified tumor DNA, Brandsma and Abramson found HPV DNA in 4 of 18 (22%) tongue and tonsillar squamous cell carcinomas, but in 0 of 3 ESCC in patients from North America.[32]

Others investigators have analyzed ESCC in patients from regions considered at moderate risk for ESCC. One PCR-based study from Japan that used L1 primers, E6-E7 consensus primers, and type-specific E7 primers, identified HPV DNA in only 3 of 45 (7%) ESCC.[18] Benamouzig et al[28] detected HPV DNA in 0 of 75 (0%) ESCC from France using HPV L1 region primers as well as type-specific primers for HPV types 6, 11, 16, 18, 31, and 33.

Thus, our results, in addition to those of others, suggest that HPV infection is infrequently associated with ESCC in patients from geographic regions at low or moderate risk of developing ESCC, such as North America, parts of Japan, and France. Perhaps other environmental factors, such as alcohol and/or tobacco use, play more important roles in esophageal squamous carcinogenesis in low and moderate risk geographic regions.[10,11] The marked difference in HPV prevalence rates for ESCC between high and low risk regions may reflect differences in the pathogenesis of ESCC in these regions. HPV infection of the esophagus in North

America may cause the development of benign epithelial proliferations, such as esophageal squamous papillomas.[33-35]

In conclusion, we have shown that HPV infection is only rarely associated with ESCC in patients from North America, a region at relatively low risk for the development of ESCC. Further studies are needed to determine if the role of HPV infection in the pathogenesis of ESCC differs between high and low-risk geographic regions.

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