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Prevalence of Human Papillomavirus in Middle Ear Carcinoma Associated with Chronic Otitis Media

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Squamous cell carcinoma of the middle ear (MESCC) is an uncommon tumor and is associated with a history of long-term chronic otitis media (COM) in most cases. Although the human papillomaviruses (HPVs) have been implicated in many human neoplasms, its role in the pathogenesis of MESCC has not been studied. Polymerase chain reaction (PCR) using consensus primers for the detection of HPV types 6, 11, 16, 18, 31, 33, 52b, and 58 was applied in screening fourteen cases of MESCC from archival material. Further subtyping was performed by restriction enzyme digestion of PCR products and by* in situ *hybridization method on tissue sections. Of the fourteen cases of MESCC, eleven were found to have HPV DNA. HPV-16 was detected in all positive cases. Five cases revealed both HPV-16 and -18. A history of long-term COM (>3 years) was found in thirteen of the cases. This is the first report to localize HPV-16/18 in MESCC on both the tissue level and the molecular level. The high prevalence of HPV in COM-associated MESCC therefore provides a good model to explain the pathogenesis of chronic-inflammation-related human malignancies. (Am J Pathol 1997, 150:1327–1333)

Squamous cell carcinoma of the middle ear (MESCC) was first recognized as a distinct entity by Politzer in 1883.[1] Although very uncommon, it is the most frequent neoplasm of the middle ear.[2,3] Its incidence has been estimated at 0.8 to 1 per 10^6 per year in the general population,[4,5] or 1 case per 8,000 to 10,000 among patients hospitalized with otological diseases.[6,7] One of the major clinicopathological features of MESCC is pre-existing chronic otitis media (COM), averaging 20 or more years in duration, in 40 to 85% of cases.[6,8–10] The mechanism responsible for the subsequent development of MESCC in COM remains elusive. Some of those carcinomas have also been associated with cholesteatomas.[11–13] Other possible causative factors include radiation therapy to the head and neck and exposure to radium.[14,15]

Human papillomavirus (HPV) has been implicated in the development of human squamous cell carcinoma in a variety of body sites covered by mucosa or skin, such as the cervix and anogenital region, as well as in carcinomas of the head, neck, and upper aerodigestive tracts.[16–18] The extent of neoplastic progression is specific to the viral type; HPV types 6 and 11 (HPV-6 and HPV-11) cause condyloma acuminata and papilloma, which generally remain benign, whereas HPV-16, -18, -33, -35, -52b, and -58 are associated with intraepithelial lesions that often progress to carcinoma.[19,20] Although neoplasms of the head and neck have been extensively investigated for the presence of HPV, the association of MESCC with HPV has not been studied yet.

In a recent study using polymerase chain reaction (PCR), Bergmann et al[21] found that 36% of the cholesteatomas contained HPV DNA that hybridized un-

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Table 1. *Summary of Clinical and Histopathological Data and the Presence of Specific Types of Human Papillomavirus in 14 Patients with Middle Ear Squamous Cell Carcinoma*

Case	Age (years)	Sex	Duration of COM (years)	Pathological diagnosis	Papillary or verrucous growth	Koilocytosis	HPV PCR	HPV ISH
1	53	M	>30	W-SCC	+	+	16	−
2	66	M	>50	W-SCC	+	+	16/18	+
3	57	M	>30	M-SCC	−	−	16	+
4	36	F	<1	W-SCC	−	−	16	−
5	61	M	>10	M-SCC	−	−	16	+
6	49	M	>40	W-SCC	−	−	−	−
7	69	M	>5	W-SCC	+	−	16/18	−
8	68	M	>10	W-SCC	+	−	16/18	−
9	61	M	>10	W-SCC	+	+	16	ND
10	66	M	>3	W-SCC	+	+	−	ND
11	55	M	>20	W-SCC	−	−	16/18	ND
12	60	F	>10	W-SCC	+	−	16/18	ND
13	53	F	>40	M-SCC	−	−	−	ND
14	46	M	>35	W-SCC	+	−	16	ND

M, male; F, female; W-SCC, well differentiated squamous cell carcinoma; M-SCC, moderately-differentiated squamous cell carcinoma; ND, not done.

der stringent conditions with a HPV-11 DNA probe. The presence of papillary growth and koilocytosis, which are characteristic of papillomavirus-induced lesions, in some cases of MESCC caused us to suspect that HPV may play a role in the development of this neoplasm. In this study, by using polymerase chain reaction (PCR) and *in situ* hybridization (ISH), we detected HPV DNA type 16/18 in 11 of 14 cases of MESCC associated with COM. This finding is significant as it provides a human model explaining the mechanism of COM and the subsequent development of MESCC.

Materials and Methods

Patients and Specimens

Fourteen cases of MESCC from the files of the Department of Pathology, National Cheng Kung University Hospital, and from the files of the Department of Pathology, Chang Gung Memorial Hospital-Kaohsiung, were used in this study. Cases with involvement of the external ear canal were included only when they were confirmed to be of middle ear origin. Biopsies were obtained at the time of diagnosis or at the time of operation. None of the patients had received treatment before the diagnosis was made. All cases were histopathologically reviewed to confirm the diagnosis and to record the presence of a papillary/verrucous growth pattern and koilocytosis.

Extraction of DNA

Two 10-μm-thick sections were cut from each paraffin block and placed in 1.5-ml Eppendorf tubes. Specimens were successively extracted with xylene and absolute ethanol, desiccated, and rehydrated with 50 μl of distilled water as previously described.[22] Each extracted specimen of DNA was amplified with β-actin primers (BA2, 5′-TACATGGCTGGGGTGTTGAA-3′, and BA3, 5′-AAGAGAGGCATCCTCACCCT-3′) to verify the existence of PCR-competent DNA.[23]

Oligonucleotide Primers

The consensus primers used here were designed by Fujinaga et al.[24] The sense primers, pU-1M and pU-31B, are located in the middle of the open reading frame E6 of the HPV genome. The antisense primer, pU-2R, is located in the middle of the E7 frame. The pU-1M/pU-2R primer pair, detecting HPV-16, -18, -31, -33, -52b, and -58, yields PCR products with 231 to 268 bp. The pU31-B/pU-2R primer pair, detecting HPV-6 and -11 only, yields 228-bp products. The primer sequences were as follows: pU-1M, 5′-TGTCAAAAACCGTTGTGTCC-3′; pU-31B, 5′-TGCTAATTCGGTGCTACCTG-3′; pU-2R, 5′-GAGCTGTCGCTTAATTGCTC-3′.

Polymerase Chain Reaction

A 50-μl volume of reaction mixture was prepared containing 1 mmol/L each of dATP, dCTP, dGTP, and dTTP, 25 pmol of each oligonucleotide primer, 0.5 U of *Taq* polymerase (Perkin Elmer, Oak Brook, IL) and 10 μl of specimen DNA in a buffer solution. The buffer solution consisted of 50 mmol/L potassium chloride, 3 mmol/L magnesium chloride, 10

mmol/L Tris/hydrochloride, pH 9.0, and 0.1% Triton X-100. The mixtures were overlaid with a drop of mineral oil. The PCR was performed in a Perkin-Elmer Cetus thermal cycler. Amplification consisted of 40 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute; however, the initial denaturation step was lengthened to 2 minutes, and the final extension step was carried out for 5 minutes. The positive controls for PCR analysis consisted of DNA from formalin-fixed, paraffin-embedded SiHa cells (HPV-16), HeLa cells (HPV-18), and HPV-6/11 carrier perianal condyloma acuminatum. Distilled water in place of the DNA templates was used as the negative control. The PCR products were either directly measured by electrophoresis on 2% agarose gel followed by staining or else broken down and analyzed by restriction enzyme digestion as described below.

Restriction Enzyme Analysis of PCR Products

PCR products (90 μl) were purified by phenol-chloroform-isoamyl alcohol (25:24:1, v/v) extraction and ethanol precipitation and suspended in 34 μl of H_2O. One-fourth of each product was digested by 3 to 4 U of *Ava*II, *Rsa*I, *Bgl*II, or *Acc*I (Boehringer Mannheim, Mannheim, Germany), respectively, in 10 μl of manufacturer-supplied buffer at 37°C for 2 hours.[24] The digested products were analyzed on 7% polyacrylamide gel (Merck, Schuchardt, Germany).

In Situ Hybridization

In situ hybridization was performed using mixed fluorescein-labeled DNA probes that were capable of detecting both HPV-16 and/or HPV-18 (Dako Corp., Carpinteria, CA). Briefly, 5-μm tissue sections were floated in a bath of distilled water onto acid-cleaned slides coated with 3-aminopropyltriethoxysilane and then heated in a 65°C incubator. They were then dewaxed in xylene, rehydrated in a graded series of ethanol washes of 100%, 95%, and 70%, and digested with pepsin/HCl (0.8% pepsin (Sigma Chemical Co., St. Louis, MO) and 0.2 N HCl) at 37°C for 10 minutes. The slides were washed in distilled water for 5 minutes twice in succession. The fluorescein-labeled DNA probes were then added, followed by placement of a coverslip and heating at 90°C for 5 minutes. The sections were then hybridized at 42°C overnight in a humid chamber. After the hybridization, the sections were soaked in Tris-buffered saline until the coverslips could be removed. The sections were subjected to a stringent wash for 30 minutes. After the wash procedure, the sections were incubated with anti-fluorescein, alkaline-phosphatase-conjugated rabbit F(ab′) in Tris/HCl buffer containing 0.01% benzalkonium chloride for 30 minutes at room temperature. The sections were then washed in Tris-buffered saline for 5 minutes and incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate solution at room temperature for 2 hours. The sections were counterstained with nuclear Fast Red for 1 to 2 minutes, rinsed in distilled water for 1 minute, dehydrated, and then mounted.

Results

Histopathology

The age at presentation varied from 36 to 69 years, with a mean of 57 years. Eleven of the patients were men and three were women. A history of long-term COM (>3 years) was documented in thirteen of the fourteen patients. Eleven cases were well differentiated squamous cell carcinoma, and three cases were moderately differentiated squamous cell carcinoma. Papillary or verrucous growth (Figure 1) was found in eight cases, of which seven were HPV positive. Koilocytosis (Figure 2) was found in four cases, of which three were HPV positive. The clinical and histopathological data are summarized in Table 1.

Detection of HPV by PCR

All DNA samples showed the expected control 214-bp β-actin band after amplification with β-actin primers and were therefore available for PCR evaluation. With the consensus primers, DNA of high-risk types of HPV were detected in 11 of 14 cases of MESCC by PCR (Table 1 and Figure 3A). HPV-16 and -18 were detected in 5 cases whereas HPV-16 alone was detected in 6 additional cases. None of the 14 cases showed HPV-6 or HPV-11 (Figure 3B). The products generated by pU-1M/pU-2R were further typed by restriction enzyme digestion. The sizes of the restriction fragments from the predicted PCR products, as reported by Fujinaga et al,[24] are shown in Table 2. Figure 4 shows one example of the results of restriction enzyme digestion of the PCR products derived from the consensus primers. Using restriction enzyme digestion analysis, all PCR products proved to be either HPV-16 or HPV-16 and -18. None of the cases presented HPV-31, -33, -52b, or -58 DNA.

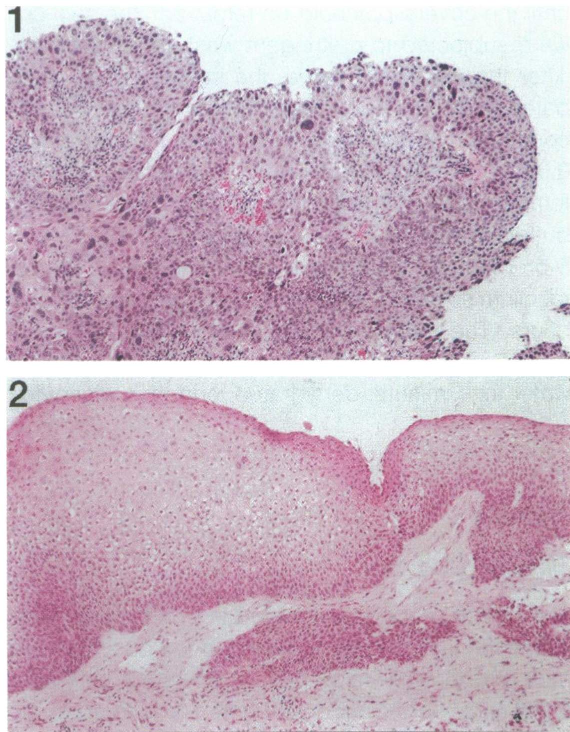
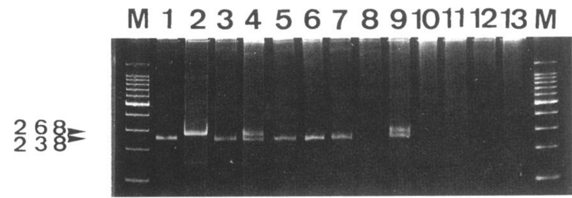


Figure 1. *Marked papillary growth pattern, exemplified here by case 1, was found in 8 of 14 cases of middle ear squamous cell carcinoma. Similar findings were found in 7 other cases. H&E stain; magnification, ×50.*

Figure 2. *Representative Koilocytosis was evident in case 9 and three other cases. H&E stain; magnification, ×50.*



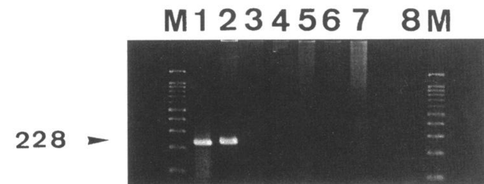


Figure 3 A: *Example of PCR detection of HPV by primer pair pU-1M/pU-2R on middle ear carcinomas.* Lane M, *100-bp size marker;* lane 1, *positive control (HPV-16);* lane 2, *positive control (HPV18);* lanes 3 *to* 9, *cases 1, 2, 3, 5, 9, 10, and 11, respectively;* lanes 10 *to* 12, *middle ear mucosa of COM;* lane 13, *negative control.* B: *Example of PCR detection of HPV by primer pair pU-31B/pU-2R on middle ear cholesteatomas and carcinomas.* Lane M, *100-bp size marker;* lane 1, *positive control (HPV 6/11 carrier perianal condyloma acuminatum);* lanes 2 *to* 4, *middle ear cholesteatomas;* lanes 5 *to* 7, *middle ear carcinomas;* lane 8, *negative control.*

In Situ Hybridization

ISH was performed on eight cases. Three of them showed positive intranuclear stain in the neoplastic cells, indicating the presence of HPV-16 and/or HPV-18. The three ISH-positive cases were also positive by PCR, whereas five ISH-negative cases evaluated by PCR were four positive and one negative (Table 1). The positively stained cells were distributed throughout the entire layer of superficial neoplastic epithelium (Figure 5) or throughout the differentiated areas of invasive tumor nests (Figure 6).

Discussion

All but one of the cases of MESCC in this series were preceded by a long-term history of COM. The frequent association of MESCC with a preceding history of COM is well known.[6,8–10] The exact pathogenesis of this association, however, remains mysterious. Although the pathology of conventional MESCC is invasive squamous cell carcinoma of various degrees of differentiation,[3,7,9] our experience has been that certain characteristic features of HPV infection, eg, papillary growth pattern and koilocytosis, are not uncommon histopathological findings. This initiated our study to investigate the presence of HPV and the roles of COM during the pathogenesis of MESCC.

Table 2. *Restriction Fragment Sizes of Consensus PCR Products*[24]

Restriction enzyme	pU-1M/pU-2R PCR products (bp)					
	HPV-16	HPV-18	HPV-31	HPV-33	HPV-52b	HPV-58
Total length	238	268	233	244	231	244
*Ara*II	157, 81	172, 96	NC	136, 108	NC	NC
*Rsa*I	NC	NC	119, 114	NC	NC	NC
Bg/II	NC	NC	NC	NC	176, 55	NC
*ACC*I	NC	NC	NC	NC	NC	126, 118

NC, no cut.

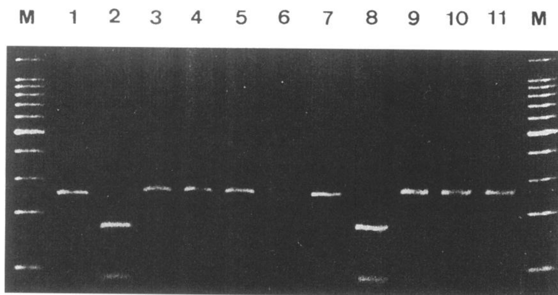


Figure 4. *Restriction enzyme analysis of PCR products of case 4* (lanes 1 *to* 5)*and case 9* (lanes 7 *to* 11) *obtained with primer pair pU-1M/pU-2R.* Lane M, *100-bp size marker;* lanes 1 *and* 7, *undigested;* lanes 2 *and* 8, *AvaII digested;* lanes 3 *and* 9, *RsaI digested;* lanes 4 *and* 10, *BglII digested;* lanes 5 *and* 11, *AccI digested;* lane 6, *negative control.*

More than 70 genotypes of human pathogenic HPV have been identified in the past 20 years. Most of them are found in benign proliferations; however, several have been discovered in malignant tumors, specifically, in cancer of cervix, other anogenital cancers, and some cancers of the skin, oral and nasal cavities, and larynx.[25] Benign papillomatous lesions of the head and neck region frequently harbor HPV-6 or -11 DNA,[26] whereas HPV-16 or -18 are present in the malignancies of the oral cavity,[27] sinonasal tract,[26] larynx,[28] and esophagus.[29] Although MESCC belongs to the head and neck region, it has not yet been linked to HPV infection. In this study we demonstrate for the first time that HPV-16/18 or HPV-16 is present in MESCC.

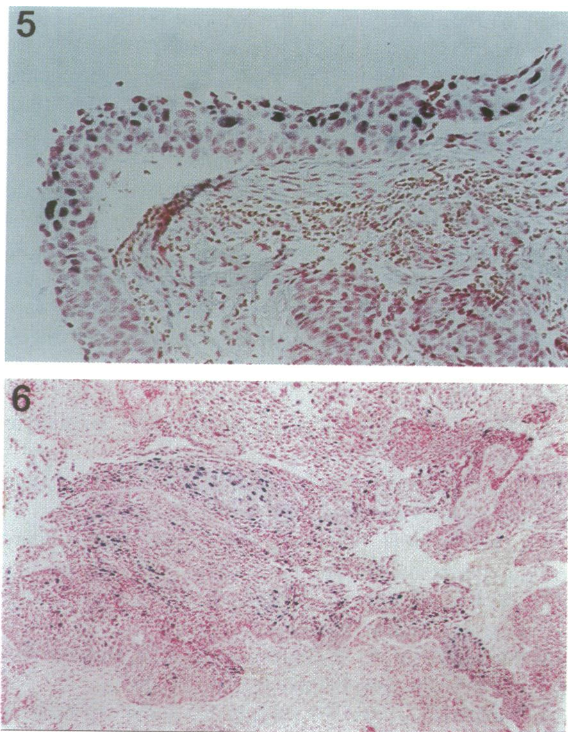


Figure 5. *Intranuclear positive stain for HPV-16/18 by* in situ *hybridization is shown scattered throughout the whole layer of superficial neoplastic epithelium. Magnification, ×80.*

Figure 6. *HPV-16/18 ISH-positive cells are more in the differentiated areas of invasive tumor nests. Magnification, ×25.*

Three of four cases with the characteristic HPV-associated pathological features of papillary growth and koilocytosis proved to be positive for HPV-16/18 or HPV-16. However, there were four HPV-positive cases with neither papillary/verrucous nor koilocytosis histopathology. It seems that this cytopathic effect is not uniformly associated with HPV infection and does not correlate strongly with the presence of the virus in MESCC.

The PCR primers used in this study are capable of detecting two types of low-risk HPV (types 6 and 11) and six types of high-risk HPV (types 16, 18, 31, 33, 52b, and 58) that are most frequently found in HPV-associated mucosal tumors. The sensitivity of this method is approximately 0.1 viral copy per cell, which is approximately the same as that of Southern blotting, and much more sensitive than the 10 to 20 copies per cell required for ISH.[24,30] It is therefore not surprising that ISH produced a smaller ratio of positive results than PCR in our study.

The types of HPV infection we detected in our study are also interesting. We found that the malignant types of HPV (16 and 18) occurred to the exclusion of all others. HPV-16 is the most prevalent virus infecting the uterine cervix and is closely associated with squamous cell carcinomas.[25] HPV-18 is most commonly associated with adenocarcinoma and small-cell neuroendocrine carcinomas of the cervix and less frequently with squamous cell carcinomas.[31,32] HPV-6 and HPV-11 are often associated with benign lesions in the head and neck region. Recently, middle ear cholesteatoma, a benign lesion also linked to MESCC,[7,33] has been shown by PCR to contain HPV-11 DNA in 36% of the cases studied.[21] Hence, the presence of HPV-6 and -11 in COM, or even MESCC, should not be unexpected. However, none of the 14 cases of MESCC in our study showed HPV-6 or -11. In a separate study, we investigated 36 cases of cholesteatoma-associated COM by PCR and ISH methods, and only one case show HPV-6/11 (submitted paper). Therefore, it seems unlikely that HPV-6 and -11 play an important role in the carcinogenesis of MESCC. The paucity of presence of HPV-6/11 in MESCC in contrast to HPV-16/18 indicate that during the carcinogenesis of MESCC initiated by COM, the infection of HPV-6/11 did not contribute to the multistep progression of tumorigenesis whereas the HPV-16/18 did.

In HPV-associated malignant transformation, viral DNA may be integrated into the cellular DNA, often

resulting in the deletion of large sectors of the viral genome. Late genes (L1 and L2) and sometimes early genes (E1 and E2) are usually lost, leaving E6 and E7 as the only open reading frames frequently found in carcinomas. The consistent transcription of E6 and E7 in tumors indicates a role for these genes in the transformed state in most cases.[34] The expression of E6 and E7 is likely to overcome the regulation of cell proliferation normally mediated by proteins like p53 and Rb, thereby allowing uncontrolled growth and providing the potential for malignant transformation.[35] Therefore, the high prevalence (79%) of the high-risk type of HPV in COM-associated MESCC in our study suggests that these subtypes play an important role in the malignant transformation from COM to MESCC. Nevertheless, the molecular mechanisms involved in the development and progression of MESCC by HPV remains to be determined.

In general, productive HPV infection is confined to the more differentiated areas in the epithelial tissue. In our cases studied by HPV ISH, the HPV DNA was present not only in the more differentiated areas (such as in the invasive squamous areas; Figure 6) but also throughout the whole thickness of the infected epithelial tissue, although not necessarily every cell (Figure 5). The easy detection of HPV DNA by PCR using consensus primers and the homogeneous nuclear staining in the neoplastic cells found by ISH are evidence of large quantities of HPV DNA and indirect evidence of HPV in the productive stage in addition to the latent phase. Primary infections with HPV probably occur as subclinical lesions in middle ear tissues with chronic infection and remain latent over long periods of time before malignant transformation. Genital HPV is usually believed to be acquired *via* sexual transmission. Blood-borne transmission of HPV has not been reported. Studies of children sharing glue pots show that common nongenital warts on fingers and hands may be transmitted by fomites. Although fomite transmission of genital types of HPV has not been demonstrated, HPV DNA has been detected on medical instruments and in laser plumes.[36,37] The genital types of HPV may be introduced into the oral cavity during oral sexual behavior and transmitted to the middle ear *via* the Eustachian tube. HPV may also be transmitted to the middle ear in patients with COM through the external ear canal by contaminated instruments.

Of the several mechanisms linked to human carcinogenesis, the association of cancers with chronic histories of trauma[38] or benign inflammatory processes[39] such as fistula tract of chronic osteomyelitis, COM, or eschar remains the most mysterious. The high association of MESCC with HPV in patients with a long history of COM therefore provides a good model to explain the pathogenesis of chronic-trauma-related human malignancies. We believe that, although cellular trauma induced by COM may be partially responsible for the malignant transformation of middle ear epithelium, during the multistep nature of cancer,[40] high-risk-type HPV infection undoubtedly plays a major role in the carcinogenesis of MESCC.

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