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Short Communication

Absence of Human Papillomavirus in Tonsillar Squamous Cell Carcinomas from Chinese Patients

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Epidemiological and experimental evidence from Western countries now consistently support an etiological role for human papillomavirus (HPV) in a subset of oropharyngeal squamous cell carcinomas (SCC), especially those originating in the tonsil. The role of HPV in the etiology of tonsil cancer in developing countries such as China has not been investigated. In this study, none of 16 tonsil cancer specimens from Chinese patients were positive for HPV DNA, whereas those from Australian patients using the same methodology gave a positivity rate of 46%. The tumors from Chinese patients, like the Australian HPV-negative subset, significantly overexpressed pRb and cyclin D1 and underexpressed p16^{INK4A} (p16). In contrast, the Australian HPV-positive cancers overexpressed p16 and had reduced expression of pRb and cyclin D1. These findings may help explain why China has a relatively low rate of oropharyngeal cancer compared with Australia. They also support the hypothesis that molecular pathways to tonsil cancer mediated by HPV are distinct from those induced by mutagens present in cigarette smoke or alcohol. ***(Am J Pathol 2003, 163:2185–2189)***

The highest incidence of squamous cell carcinoma (SCC) of the oropharynx in 1990 was reported in Western Europe and Australasia, with rates of 28.6 and 22.5 per 100,000 in males and 5.2 and 7.5 per 100,000 in females, respectively.[1] The lowest rates, of approximately 4 per 100,000 in males and 2 per 100,000 in females, were recorded in developing countries such as China. These discrepancies have generally been attributed to cultural or social factors affecting high-risk practices, such as tobacco smoking or chewing and alcohol consumption, or to dietary or genetic factors.[2] However, differences in exposure to oncogenic viruses, such as human papillomavirus (HPV), may well be a contributing factor. The association between HPV and oropharyngeal cancer is strongest in the tonsil. HPV-positivity rates in tonsil cancer from high incidence countries have ranged from 45% to 70%, with HPV 16 invariably predominant.[3–7] The extent of involvement of HPV in tonsil cancer in low incidence countries, such as China, is unknown.

We have recently analyzed formalin-fixed, paraffin-embedded tonsil cancers from Australian patients for human papillomavirus (HPV) DNA by polymerase chain reaction (PCR) and sequencing; and assessed the level of expression of key cell cycle proteins pRb, p16^{INK4A} (p16), cyclin D1, p53, p21$^{CIP1/WAF1}$ (p21), and p27^{KIP1} (p27) by semi-quantitative immunohistochemistry.[8,9] Approximately half of the tumors were found to contain HPV DNA, almost all type 16. Our findings supported growing evidence of a strong association between HPV-positivity and overexpression of p16 and reduced expression of cyclin D1 and pRb.[6,7,10] Conversely, HPV-negativity was associated with loss of p16 expression and up-regulated pRb and cyclin D1. In some studies of oropharyngeal cancers, HPV-positivity and p53 mutations have been reported to be almost mutually exclusive,[11] but in others they have been found to co-exist.[12] Although we did not find any relationship between HPV status and p53 expression, the p53 antibody used was not mutation-specific. There was no association between HPV status and p21 or p27 expression, despite their reported interactions with HPV 16 oncoproteins (E6/E7) and the transactivation of p21 by p53.[13,14] We have subsequently shown that

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patients with HPV-positive tonsil cancers have less risk of recurrence and better survival prospects than HPV-negative cancers.[9] Collectively, these findings support other experimental and epidemiological data suggesting that HPV-positive tonsil cancers are biologically distinct from those that are HPV-negative.[3,7,15]

The major aim of the present study was to determine whether differences in HPV-positivity rates might help explain the wide discrepancies in the incidence of oropharyngeal cancer between China and Australia. Assessment of the levels of expression of proteins in the p53 and pRb pathways was carried out to provide data on the molecular mechanisms underpinning HPV-related and -unrelated tonsil cancer in persons of Chinese ethnicity. In addition, analysis of the histological features of tumors from Chinese and Australian patients was made in an attempt to identify relationships between histology, country of origin, and HPV status of the tumors.

Materials and Methods

HPV Typing and Immunohistochemistry for Expression of Cell Cycle Markers

Investigations were carried out on formalin-fixed, paraffin-embedded tonsil cancers from 16 patients treated at the First and Second Teaching Hospitals of Jilin University (in Jilin province in northeast China) between 1973 and 2002. There were no selection criteria other than the availability of tumor material for the analyses. Approximately 40 patients were initially identified from the Medical Records Department of the two hospitals as having had biopsies of SCC of the tonsil taken over this period. Paraffin blocks of tumor for 23 patients were located in the archives. Sixteen of these 23 blocks were found to contain adequate amounts of residual tumor on hematoxylin and eosin (H&E) section. There was no evidence of a pattern in terms of demographics or time frame for tumors unavailable for examination. The methodology was identical to that used for previous analyses of the tonsil cancers from Australia,[8,9] except that an additional consensus PCR (FAP59/64)[16] was carried out, and the p53 antibody used was mutation-specific. Briefly, tumor lysates were tested for HPV using two type 16-specific PCRs, plus four consensus PCRs or nested/consensus PCRs (GP5+/6+,[17] CP65/70ct-CP66/69ct,[18] FAP59/64,[16] and A5/A10-A6/A8[19]) that collectively detect a broad spectrum of mucosal and cutaneous HPVs in paraffin-embedded tissues. A β-globin PCR[20]was used to confirm the presence of amplifiable DNA in all specimens. The controls and precautions used to prevent cross contamination were as previously published.[21] Immunohistochemistry was carried out on 5-μm sections using monoclonal antibodies p53, p21, p27, pRb, and cyclin D1 (PAb240, SX118, SX53G8, Rb1, and DCS-6: all from DAKO, Carpinteria, CA) and p16 (Ab-4, Clone 16p04 Neomarkers, Fremont, CA). Sixty-seven tonsil cancers from Australian patients of known HPV status and cell cycle marker expression[9] were retested for p53 using the mutation-specific antibody. Overall data from Chinese and Australian cohorts were compared using the Mantel-Haenszel method[22] and Pearson's chi-squared test. Only P values of <0.05 were considered significant. A comparison of the mean age of the two groups was carried out using Student's t-test.

Histological Features of the Tumors

Grading of the Chinese and Australian tumors was evaluated in a blinded manner by two observers according to the World Health Organization criteria for squamous cancers of the oral mucosa.[23] Forty-eight of the 67 Australian tumors were resected specimens and 19 were pretreatment incisional biopsies. For the Chinese cases, only pre-radiation incisional biopsies were available. Individual histological features evaluated included predominant architectural growth pattern (sheets, nests, or islands), presence and amount of extracellular keratin, nuclear pleomorphism, and mitotic counts. With regard to growth patterns, sheets were defined as confluent masses of tumor cells >2 mm in diameter with no intervening stroma; islands were cohesive groups of tumor cells measuring 0.5 to 2 mm in diameter separated by stroma; and nests were cohesive groups of tumor cells <0.5 mm in diameter. Keratin was graded as minimal (<10% tumor) or moderate (10 to 25% tumor), or abundant (>25% tumor). Nuclear pleomorphism was graded as minimal, moderate, and severe. Mitotic counts were based on assessments of five high-power (×400) fields in the most mitotically active area. The presence of precursor lesions was recorded.

Results

Characteristics of the Chinese Cohort

Personal characteristics and experimental data are shown in Table 1. None of the 16 Chinese tumors were positive for HPV DNA by either the HPV 16 type-specific or the consensus PCRs, although all samples gave strong positive signals in the β-globin PCR. The majority of tumors from China showed up-regulated expression of pRb, cyclin D1, and p21, down-regulated expression of p16 and p27, and positive staining for p53. There was no evidence of any relationship between patient age, gender, or tumor differentiation and cell cycle protein expression in this small sample.

Overall Comparisons between Chinese and Australian Cohorts

The overall relationships between the 16 Chinese and the 67 Australian cases are shown in Table 1 ("p1" values). There were proportionally more females than males in the Chinese *versus* the Australian group, but the difference was not significant. There was no significant difference in mean age between the two groups of patients and the age range was similar. There was a significant difference in HPV-positivity rates in the tumors of the two groups (0 of 16 *versus* 31 of 67). Tumors from Chinese patients were

Table 1. Comparison of Demographics, HPV Status, and Cell Cycle Marker Expression in Australian and Chinese Tonsil Cancers

	Chinese *N* = 16	Australian *N* = 67			p Value**		
HPV status	Negative *N* = 16	Total *N* = 67	Negative *N* = 36	Positive* *N* = 31	p1 0.001	p2	p3
Demographics							
Gender (M:F)	11:5	51:16	28:8	23:8	NS	NS	NS
Mean age (years)	56	57	61	52	NS	0.062	NS
Age range	37–72	35–81	45–81	35–72			
Cell cycle marker expression							
p16 Up	6%	49%	17%	87%	0.004	NS	<0.001
pRb Up	71%	49%	82%	19%	NS	NS	0.003
Cyclin D1 Up	75%	42%	73%	13%	0.07	NS	<0.001
p53 Positivity	63%	52%	53%	52%	NS	NS	NS
p21 Up	92%	63%	76%	65%	0.09	NS	NS
p27 Up	21%	27%	32%	41%	NS	NS	NS

*, 30 of 31 Australian HPV-positive tumours contained HPV16.
**, p1, Chinese *versus* all Australian tumours; p2, Chinese *versus* Australian HPV-negative subset; p3, Chinese *versus* Australian HPV-positive subset.
N, number patients; NS, not significant, $p > 0.1$; Up, up-regulated expression.
Comparisons between HPV-positive and -negative cancers from Australian patients are as published.[8]

significantly less likely to have up-regulated p16 expression than their Australian counterparts, but there were no other significant differences in the expression of cell cycle markers.

Associations between HPV Status and Country of Origin

In view of current theories that HPV-positive and -negative cancers are separate biological entities, associations were also sought between HPV status and country of origin. There were no significant differences in mean age or gender ratio between the Chinese and either the HPV-negative or -positive Australian subgroup (Table 1 "p2, p3" values, respectively). The strong association between overexpression of pRb, cyclin D1, and reduced expression of p16 seen previously in the Australian HPV-negative tumors[9] was again a feature of the Chinese tumors (all of which were HPV-negative). Predictably, p16 was significantly overexpressed, and pRb and cyclin D1 had reduced expression in the Australian HPV-positive subgroup compared with the Chinese group. The lack of association between HPV status and p53 expression in Australian tumors previously reported[9] was confirmed here using the mutation-specific antibody. In fact, the proportion of tumors from Australian patients showing aberrant p53 expression was almost the same in the HPV-positive and -negative subgroups. There were no

Table 2. Comparison of Individual Histological Features of the Tumours in Relation to Country of Origin and HPV Status

	Chinese *N* = 16	Australian *N* = 67		
HPV status	Negative *N* = 16	Total *N* = 67	Negative *N* = 36	Positive *N* = 31
Tumor differentiation*				
Well	31%	10%	8%	13%
Moderate	31%	55%	61%	48%
Poor	38%	34%	31%	39%
Predominant growth pattern				
Sheet	56%	33%	31%	35%
Nest	44%	6%	9%	3%
Island	0%	38%	40%	35%
Mixed	0%	23%	20%	26%
Amount of extracellular keratin				
Mild	75%	59%	46%	74%
Moderate	19%	24%	34%	13%
Abundant	6%	17%	20%	13%
Nuclear pleomorphism				
Mild	19%	5%	3%	6%
Moderate	44%	61%	74%	45%
Severe	38%	35%	23%	48%
Mitotic counts				
Mean (range)	16.19 (5–49)	14.3 (2–57)	12.0 (2–21)	16.8 (2–57)

*, differentiation according to World Health Organization criteria for SCC of aerodigestive tract.[23]

significant relationships in the levels of p53, p27, or p21 expression between the Chinese and either of the Australian subgroups.

Associations between Tumor Histology, Country of Origin, and HPV Status

Overall differences between tumor differentiation and country of origin were not statistically significant (P = 0.068). However, when the different grades of differentiation were considered individually, Chinese patients were four times more likely than their Australian counterparts to have well-differentiated tumors [OR = 3.9, CI = (1.05–14.5)], and this trend was independent of HPV status. With regard to individual histological variables, Chinese tumors tended to be well organized, featuring sheets and nests rather than islands or individual cells, whereas the Australian tumors were more likely to be disorganized irrespective of HPV status. The lack of keratin in the majority of Chinese tumors was inconsistent with a more differentiated phenotype but the biopsies examined may not have been representative of the entire tumors. Chinese tumors tended to have less nuclear pleomorphism than their Australian counterparts; mitotic counts were similar. No precursor lesions were seen in any of the tumors from either country. The results comparing Australian incisional biopsies with the Chinese samples (all incisional biopsies) and Australian incisional biopsies with Australian resected specimens were consistent with overall findings (data not shown). Among the Australian cases, there was no significant association between tumor differentiation and HPV status. However, analysis of the individual histological features showed that HPV-positive tumors were more likely to have higher mitotic counts than their HPV-negative counterparts, and there were strong trends toward greater nuclear polymorphism and less keratinization.

Discussion

Variations in HPV-positivity rates in tonsil cancers across the published surveys (45% to 70%) have generally been attributed to the methodology for HPV detection. The lack of HPV in the cancers from Chinese patients was of particular interest. It is unlikely that the fixation processes in China adversely affected subsequent HPV DNA amplification since all of the Chinese cancers were strongly positive for chromosomal β-globin. Moreover, the associated expression patterns for p16, cyclin D1, and pRb were entirely consistent with those previously reported in our Australian HPV-negative tonsil cancers. It is also unlikely that the tumors contained virus at undetectable levels, or that novel HPV types were involved, since the HPV detection system involved a combination of sensitive and wide-ranging assays. Data relating to tumor stage and node status of the Chinese cohort were not available. However, the male to female ratio was consistent with a previous report of oropharyngeal cancers from China,[1] and the median age (in the sixth decade) was consistent with data from other countries. These figures suggest that the sample analyzed in this study, although small, is genuinely representative. Therefore, our findings imply that the involvement of HPV in tonsil carcinogenesis may vary along ethnic lines.

There are at least two possible explanations for the marked difference in HPV-positivity rates between China and Australia. Since HPV 16 is overwhelmingly the most common papillomavirus found in tonsil cancers, the levels of HPV 16 circulating in some areas of China may be relatively low. Indirect evidence supporting this theory has come from the lower incidence of cervical cancer (for which HPV 16 is the major risk factor) reported in China compared with Australia (4.4 *versus* 12.9 per 100,000 in 1990).[1] Epidemiological studies have shown that exposure to HPV increases the risk of oropharyngeal cancer,[24,25] suggesting that the virus associated with tonsil cancer may be sexually transmitted, although this has not been confirmed. Hence a lower rate of oral exposure to potentially oncogenic HPVs could be due to cultural or social prohibitions that affect sexual practices, notably oro-genital contact. Clearly, HPV status does not fully account for the difference in incidence of oropharyngeal cancer between China and Australia. It is assumed that the difference in prevalence of cigarette smoking could represent a major additional factor. In many developing countries, such as China, cigarette smoking has only become widespread over the last 30 years, so that the main consequences will not emerge for several decades.[26] Even so, the major diseases attributable to tobacco smoking are reportedly very different in China compared with the U.S., and within China between one city and another.[26]

The relationship between HPV-positivity and p53 mutations in oropharyngeal cancer remains controversial, although the findings of this study suggest that they can co-exist. Up-regulated cyclin D1 and loss of the p16 protein in association with intact pRb has been reported in many human cancers.[27] In contrast, HPV-positive cancers seem to be characterized by reduced expression of pRb and cyclin D1 in the context of high levels of p16, a scenario pointing to a feedback loop between pRb, cyclin D1, and p16. The HPV 16 E7 oncoprotein binds to the same site as cyclin D1 on pRb, and it has been suggested that E7 overcomes the need for cyclin D1 in the G1 phase of the cell cycle.[3,7,15]

To our knowledge, this is the first study comparing the histology of tonsil cancers from Western and Asian countries. We provide evidence of differences in differentiation, and specifically in growth patterns, that may be independent of the HPV status of the tumors, but larger studies of resected tumor specimens will be needed for clarification. We were unable to support a significant association between HPV-positivity and poor tumor differentiation reported in some previous studies[6,7] but not others.[28,29] However, there was evidence of a relationship between HPV-positivity and individual markers of a poorly differentiated phenotype, notably minimal keratinization, increased nuclear polymorphism, and high mitotic count.

This study provides the first evidence that HPV currently has virtually no role in the etiology of tonsil cancer

in China. Large multi-center studies from China and other low incidence regions, such as Africa or Central America, will be needed for confirmation. The findings of this study also provide further support for a role of HPV as an etiological agent for a subset of tonsil cancers.

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