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Presence of Human Papillomavirus in Dentigerous Cyst and Ameloblastoma

(HPV in Dentigerous Cyst and Ameloblastoma)

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ABSTRACT

Aims: The aim of the current study was to detect and compare Human papillomavirus (HPV) in dentigerous cyst and ameloblastoma.

Materials and methods: The study included 32 samples, which distributed into dentigerous cysts (n=16) and ameloblastomas (n=16). Conventional PCR assay using SPF1/2 consensus primers and immunohistochemical analysis were performed for the detection of HPV–DNA and HPV–L1 protein respectively.

Results: HPV–DNA was detected in 25% (4/16) of dentigerous cyst samples and in 45.45% (5/11) of ameloblastomas samples, whilst HPV–L1 protein expression was identified in 25% (4/16) of dentigerous cysts and in 75% (12/16) of ameloblastomas. Chi–square test showed significantly higher expression of HPV–L1 protein in ameloblastomas than in dentigerous cysts ($P<0.01$). Kappa coefficient showed moderate agreements ($k=0.526$) between PCR and immunohistochemical results.

Conclusions: The current study concludes that both HPV–DNA and HPV–L1 protein could be detected in ameloblastomas and to a lesser extent in dentigerous cysts. This may indicate a possible relation of HPV with those lesions.

Keywords: dentigerous cyst**;** ameloblastoma**;** human papillomavirus; HPV; PCR**;** Immunohistochemistry

INTRODUCTION

Dentigerous cyst is an odontogenic lesion which represents the second most common odontogenic cyst, accounting for approximately 24% of all true cysts in the jaws and occurs in association with unerupted tooth [1, 2]. Small dentigerous cysts are usually completely asymptomatic and discovered on a routine radiographic examination. However, dentigerous cysts can grow to a considerable size resulting in facial asymmetry[2].

Ameloblastoma represents the second most common odontogenic tumor[3], accounting for approximately 1% of all tumors and cysts of the jaws[4]. It is slow–growing, locally invasive, and has a high recurrence rate if not treated adequately[3]. Three clinicoradiographic situations of this tumor have been identified, including the solid or multi–cystic variant (86% of all ameloblastomas), the unicystic variant (13% of all ameloblastomas), and the peripheral variant (1% of all ameloblastomas)[2].

Theoretically, ameloblastoma may arise from the rests of dental lamina, developing enamel organ, the epithelial lining of the odontogenic cyst, or the basal cells of the oral mucosa[2]. Nevertheless, the lining of dentigerous cyst derived from reduced enamel epithelium[5]. The etiology and exact histogenesis of dentigerous cyst and ameloblastoma are unknown[5, 6]. Factors such as trauma, tooth extraction, infection, a past history of an endodontically treated tooth, virus[7], and genetic causes[8] have been associated with the etiopathogenesis of ameloblastoma. Most authors favor a developmental origin from a tooth follicle as possible histopathogenesis of dentigerous cyst. However, inflammatory origin as a result of inflammation from a non–vital deciduous tooth or other source spreading to involve the tooth follicle was also considered[5].

Human papillomaviruses (HPVs) are a group of small, nonenveloped, double–stranded DNA tumor viruses with about 7900 nucleotide bases long. Approximately 200 types of HPV have been identified[9, 10]. HPVs can be clinically classified as low–risk, which is associated with benign epithelial proliferation, and high–risk, which is associated with malignant tumor[9]. The HPV genome contains eight open reading frames (ORFs), which are potential coding sites of

six early proteins (E1, E2, E4, E5, E6, and E7) and two late proteins (L1 and L2)[10]. HPV is nearly ubiquitously present in humans, but ultimately only a small proportion of infected individuals develops cancer[11]. The specific role of HPV in the development of premalignant and oral squamous cell carcinoma continues to be debated topic[12]. The HPV E6 and E7 oncoproteins contribute to tumor initiation and also play important roles in malignant progression through the induction of genomic instability and other mechanisms[9].

Only a few studies have been conducted for the detection of HPV presence in ameloblastoma and keratocystic odontogenic tumor. The aim of the current study was to detect and compare the presence of HPV in dentigerous cyst and ameloblastoma.

MATERIALS AND METHODS

Patients and Samples

A total of 32 odontogenic lesions were used; 28 samples of them were formalin fixed paraffin embedded (FFPE) samples, retrieved from the archive of Oral Pathology Laboratory, Tongji Hospital, Huazhong University of Science and Technology, over the years (2008–2010). The remaining four samples were frozen tissue samples that were prospectively collected from patients intended maxillofacial surgery at Tongji Hospital, Huazhong University of Science and Technology during the research time. Overall, the study samples were distributed into dentigerous cysts that included 16 samples (13 FFPE and three frozen) and ameloblastomas which included 16 samples (15 FFPE and one frozen). There was no true tissue equivalent exists to serve as a negative control, as both dentigerous cyst and ameloblastoma represent odontogenic lesions that encompass reactive tissues and tumors which replacing the healthy bone. All studied ameloblastomas were of central multi–cystic type. This type involved different histopathological subtypes that imposed on two main variants which are the follicular and plexiform subtypes. Several authors have reported no correlation between the histologic pattern of central multi–cystic ameloblastoma and its clinical behavior[4, 7, 13]. In addition, some samples expressed multiple variants of multicystic ameloblastoma in one slide. For all those reasons, different histopathological variants of multi–cystic ameloblastomas were dealt as same in the current study. All patients were free from the acquired immune deficiency syndrome (AIDS) and did not take HPV vaccine, or any antivirus or immunosuppressive therapies. This study was approved by our Institutional Review Board. The Declaration of Helsinki protocols were followed during the whole study.

PCR Analysis

Extraction and Quality Assessment of the DNA: Each FFPE sample was cut into five pieces of 10 µm thick sections and deparaffinized using xylene then rehydrated by subsequent descending concentrations of ethanol and distilled water. After that, DNA was extracted using DN32 DNA extraction kit (Aidlab. Co. LTD, Beijing, China) which based on the silica membrane extraction method and according to the manufacturer instructions. Nevertheless, several represented pieces for a total weight of 30 mg were used for DNA extraction from each frozen tissue sample. Aliquot of EDTA, nuclear lysate solution, and proteinase K solution were added to the specimen pieces and placed at 55 ºC in water bath overnight and then the DNA was extracted according to the manufacturer instructions of the used DN08 DNA extraction kit (Aidlab, Co. LTD, Beijing, China). In order to verify the quality of each extracted DNA, 3 µl of isolated DNA solution from each sample was amplified with primers recognizing the albumin gene. Consequently, five FFPE samples of ameloblastoma were failed to reveal an integral genomic DNA and their PCR results of the HPV–DNA detection were excluded.

Amplification Reaction and Visualization of the Final PCR Products: The prevalence of HPV–DNA was evaluated by conventional PCR method using the broad–spectrum SPF1/2 HPV primers which located in the L1 open reading frame as described previously[14]. The PCR reaction was performed in a final reaction volume of 25 µl containing 3 µL of the isolated DNA solution, 12.5 µl of 2 X Taq MasterMix (Aidlab Co. LTD, Beijing, China), 200 mmol/L each deoxynucleoside triphosphate, and 15 pmol of forward and reversed primers. The thermal profile of the PCR was: one cycle of activation for 9 min at 94 ºC followed by 60 cycles of amplification, each cycle consisted of 30 sec at 94 ºC, 45 sec at 52 ºC, and 45 sec at 72 ºC. The final extension step was 5 min at 72 ºC. Each PCR experiment was performed with positive and negative controls. The 65–bp PCR products were run through 3% agarose gel and visualized with ethidium bromide staining.

Immunohistochemistry

Immunostaining was performed using the standard Streptavidin–biotin peroxidase complex method and kit (Boster Biological Technology, China). FFPE samples were cut into 5 µm thickness tissue sections and dewaxed, rehydrated then endogenous peroxidase was quenched in 3% H_2O_2, followed by antigen unmasking in 0.01 M citrate buffer that heated to the boiling point in a microwave oven. The frozen samples were cut into 5 µm thick sections in the cryostat

chamber (Leica Biosystem Nussloch GmbH, Germany), then dried at room temperature and fixed in 4% paraformaldehyde. The endogenous peroxidase activity was blocked using 0.03% H_2O_2 in absolute methanol. After that, both FFPE and frozen samples were equally treated with normal goat serum for 50 min at room temperature. Subsequently, the sections were incubated at 4°C for overnight with 1:100 diluted polyclonal rabbit anti–HPV that was raised against a synthetic peptide corresponding to the N–terminus of L1 protein (Beijing Biosynthesis Biotechnology Co, China). After that, the sections were treated with 10 µg/ ml biotinylated secondary antibody (goat anti rabbit IgG) for two hours at room temperature. Then, they were stained with 20 µg / ml SABC– peroxidase complex. Finally, the sections were developed with 3,3'–Diaminobenzidine substrate and slightly counterstained with Mayer's hematoxylin. Negative controls were incubated by PBS instead of the primary antibodies.

HPV–L1 immunoreactivity was assessed using a light microscope. The positive results were interpreted as yellowish–brown precipitates, which predominantly intranuclear, in a mainly focal, but some diffused pattern, whereas the negative results were indicated by the lack of precipitate intracellularly.

Statistical Analysis

The statistical analysis was performed with SPSS–Windows v.19.0 (SPSS Inc., Chicago, IL, USA). Chi–square test and Kappa statistics were used in analyzing the data. Differences were deemed statistically significant if ($P<0.05$).

RESULTS

The most relevant clinicopathological features are summarized in Table 1. HPV–DNA was detected in 25% (4/16) of dentigerous cyst samples and in 45.45% (5/11) of ameloblastoma samples (Figure. 1). HPV–L1 protein was immunohistochemically detected as a yellowish– brown stain that was mainly expressed in the nucleus of positive odontogenic epithelial cells in dentigerous cysts and ameloblastomas (Figure. 2). The expression of HPV–L1 protein was higher in the stellate reticulum–like cells than in the columnar cells of ameloblastomas (Figure. 2A). Some samples of dentigerous cyst and ameloblastoma showed koilocytic features in their odontogenic epithelium. However, not all koilocytes expressed HPV–L1 staining. A positive HPV–L1 protein expression was identified in 25% (4/16) and 75% (12/16) of dentigerous cysts and ameloblastomas respectively. Statistical analysis using chi–square test showed significant higher expression of HPV–L1 protein in ameloblastomas than in dentigerous cysts ($P<0.01$). The Kappa coefficient showed moderate agreements ($k=0.526$) between PCR and immunohistochemichal results.

Table 1 Clinicopathological features of the studied cases.

Studied cases	n	male	female	Mean age	Mandible	Maxilla
Dentigerous cyst	16	13	3	39.69	4	12
Ameloblastoma	16	9	7	33.56	15	1
Total	32	22	10	36.62	19	13

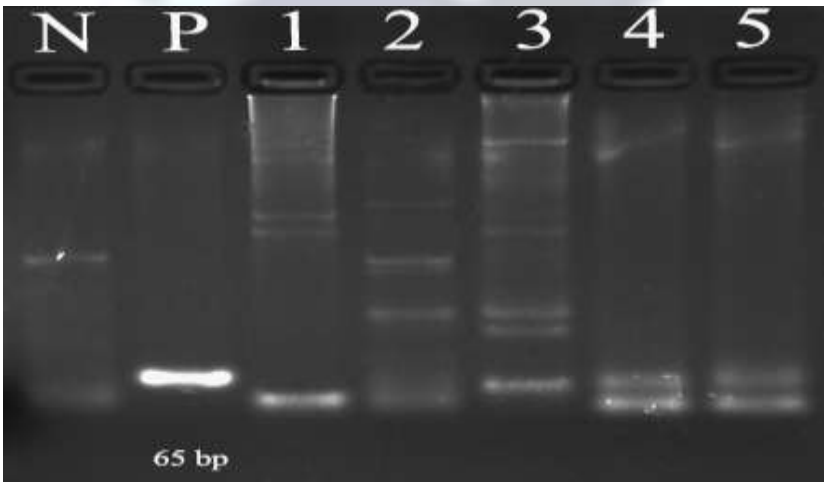

Figure. 1 Visualization of PCR products for HPV–DNA in FFPE and Frozen tissue samples of dentegerous cysts and ameloblastomas. The 65 bp PCR products size are visualized on 3% agarose gel that stained with ethidium bromide. (N) Negative control; (P) positive control; (1, 2) are the negative results. Nevertheless, (3, 4, and 5) represent the positive results.

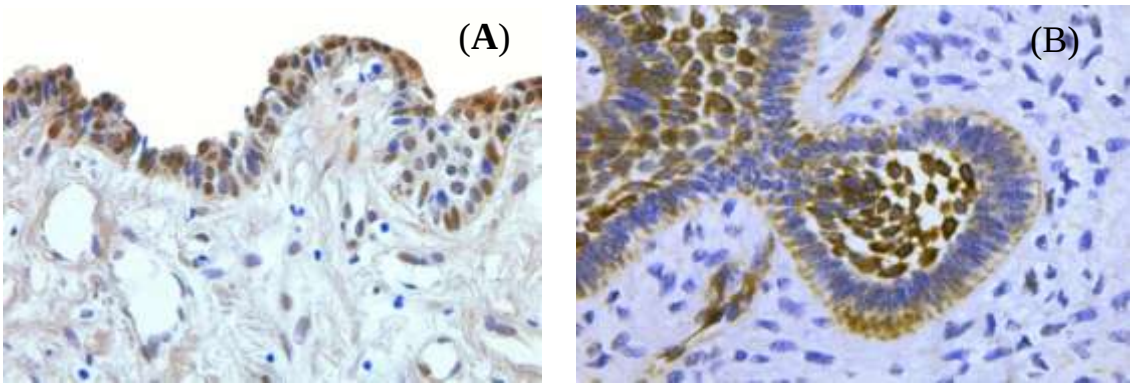

Figure 2: Immunohistochemical staining of HPV–L1 protein in dentigerous cyst and ameloblastoma (×400). (A) Dentigerous cyst: yellowish–brown nuclear staining of the HPV–L1 immunopositive cells present through the cystic wall; (B) multicystic ameloblastoma: HPV–L1 yellowish–brown nuclear immunostaining is predominantly noted in the central stellate reticulum like cells.

DISCUSSIONS

HPV is nearly ubiquitously present in humans, but ultimately only a small proportion of infected individuals develops cancer[11]. HPV–DNA was discovered in the saliva of healthy individuals[15]. Both mucosal and cutaneous HPVs may infect the epithelium of the upper aerodigestive tract[16]. Nonsexual oral infection through salivary or cross transmission is plausible[17]. The specific role of HPV in the development of premalignant and oral squamous cell carcinoma continues to be debated topic[12]. The HPV E6 and E7 oncoproteins contribute to tumor initiation and also play important roles in malignant progression through the induction of genomic instability and other mechanisms[9].

The current study investigated and compared the presence of HPV in dentigerous cyst and ameloblastoma using PCR and immunohistochemical analyses for the detection of HPV–L1 protein and HPV–DNA respectively. The PCR and Immunohistochemistry results are comparable, where kappa coefficient test showed a moderate agreement between the two testing methods (k= 0.526). There is no consensus regarding the most appropriate method for detecting HPV in the tissue sample[18]. However, PCR is widely used in research settings. HPV–L1 capsid protein is expressed in the active phase of the viral infection and is necessary in viral cellular cycle completion. Consequently, viral protein detection, by immunohistochemical reaction is an evidence of active HPV infection in the examined tissues[19].

The etiology of ameloblastoma has not been elucidated till now. Factors such as trauma, tooth extraction, infection, a past history of an endodontically treated tooth[7], and genetic causes[8] have been associated with the etiopathogenesis of ameloblastoma. The possibility of viral role in the pathogenesis of ameloblastoma has been investigated since 1960s; where the preliminary studies concentrated on the role of polyoma virus in the development of ameloblastoma[20, 21].

The first investigation relating HPV with ameloblastoma was performed by Kahn[7], who demonstrated the presence of HPV in ameloblastomas of young persons. Subsequently a relatively few studies were conducted to detect the presence of HPV in ameloblastoma[6, 22–26] and keratocystic odontogenic tumors[27, 28]. The results of these studies were greatly inconsistent. Some results did not confirm the presence of HPV[25, 28], while the others confirmed the presence of different HPV genotypes using different investigation methods in both ameloblastomas and keratocystic odontogenic tumors[6, 7, 22–24, 26, 27]. This controversy could be attributed to the methods of samples collection, quality of the available samples, or the methodology used. Another possibility is the different ethno–geographic origins of the subjects examined, as one property of the virus related neoplastic disorder is the difference in different geographic areas[29]. Accordingly, all the samples used in the current study were taken from the Chinese patients. Studies confirmed the presence of HPV in the odontogenic tumors also have different conclusions regarding the role of the virus in the involved lesion. While some of them regarded HPV as an etiological factor or cofactors in the development of the ameloblastomas and keratocystic odontogenic tumors[6, 7, 22, 26], others regarded it as a secondary infection resulted from contact between the tumor epithelium and the oral mucosa[23], or due to contamination from the surface mucosal epithelium induced by the surgical manipulation[24].

Koilocytes were noted in some present study samples. However, not all koilocytes expressed HPV–L1 staining, indicating that only some koilocytes have the complete structural assembly of the viral capsid protein. This finding is in

agreement with another investigation which showed that koilocytes were found in all HPV positive and HPV negative unicystic ameloblastomas and in one of the two positives solid ameloblastomas[26]. The HPV–L1 expression in the current study was more in the central stellate reticulum–like cells than in peripheral columnar cells of ameloblastoma. This was in disagreement with the study of Kahn[7], who found predominant HPV–L1 positivity in the peripheral columnar cells.

The wall of dentigerous cyst represents a possible origin of the ameloblastoma[2], Previous studies[7, 26] confirmed the presence of HPV in intrabony ameloblastoma and speculated that HPV presence could be derived from the epithelial lining of dentigerous cyst[26], or implied the neoplastic transformation of the dentigerous cyst odontogenic epithelium to ameloblastoma[7]. However in the lack of PubMed published articles that dealt with the presence of HPV in dentigerous cyst, we investigated the presence HPV–DNA and HPV–L1 capsid protein in dentigerous cysts and compared it with that of ameloblastomas. Consequently, the results demonstrated that 25% of dentigerous cysts revealed the presence of HPV–DNA or HPV–L1 in their odontogenic epithelium, which was significantly less than that of ameloblastomas. These findings may reflect the higher association of HPV with ameloblastoma than with dentigerous cyst and could support the theory of ameloblastoma development from the wall of the dentigerous cyst.

Cox et al stated that cancer, keratosis, and cystic lesions merely provide the correct type(s) of differentiating cell in which a previously latent HPV may replicate[27]. This fact may explain the current study results of HPV–L1 protein expression in the samples of dentigerous cyst.

The exact histogenesis of dentigerous cysts remains unknown, but most authors favor a developmental origin from a tooth follicle. Ben et al concluded that at least two types of dentigerous cysts are existed. The first type is developmental in origin, while the second type is of inflammatory origin and occurs in immature teeth as a result of inflammation from a nonvital deciduous tooth or other sources which spread to involve the tooth follicle[5]. HPV in turn, may represent a possible source of tooth follicle inflammation or it may infect the already inflamed tooth follicle, as it was found that an association between chronic inflammation and HPV infection is biologically plausible and supported by multiple epidemiologic and molecular studies of cervical HPV infection[30].

Further studies are needed to evaluate a possible etiological role of HPV in the development of dentigerous cyst. Interestingly, Xuan et al. demonstrated a relation between the expression of sonic hedgehog (SHH) and HPV16 infection in cervical cancer[31]. Knowing that SHH signaling molecules have been demonstrated in both dentigerous cyst[32] and ameloblastoma[8], one could speculate that HPV may have a possible role in dentigerous cyst and ameloblastoma.

Both low and high risks HPV genotypes were found in ameloblastomas[6, 22–24, 26]. However, a significant number of undifferentiated HPV genotypes were also noted[6, 24, 25]. In addition, many HPV genotypes were not fully sequenced till now. Our study detected the presence of consensus HPV–DNA and HPV–L1 protein. Larger samples studies are needed to specify the specific genotype(s) in dentigerous cyst and ameloblastoma.

A Predicament in explaining the mechanism by which HPV could enter through the bone and infect the odontogenic epithelium is the feature of the previous studies that confirmed the presence of HPV in intrabony odontogenic lesions. Accordingly, some studies excluded that possibility, attributing HPV presence to secondary infection from the contacted oral mucosa or contamination during surgery[23, 24]. However, Kahn[7] concluded a possibility of HPV to be acquired in utero or at parturition and involves the invaginating primitive enamel organ, then at a later date, through complex sequences of events, the virus stimulates growth factors or reduces the growth factor's natural inhibitory control.

Bodaghi et al. proposed that peripheral blood mononuclear cells may regarded as HPV carriers and might spread the virus through blood, suggesting that those cells may migrate to sites of HPV infection and take up HPV from tissues or the bloodstream as they do for many other viral infections. Consequently, peripheral blood mononuclear cells might serve as a source of HPV infection to the epithelial cells and contribute to their nonsexual spread[33]. Taking together that HPV–DNA was also detected in the sera of patients with head and neck squamous cell carcinoma[34]. As well, HPV–DNA was observed in deep organs like prostatic tissue[35] and breast cancer tissue[36]. These observations could further explain the possibility odontogenic epithelium involvement in HPV infection, speculating that blood circulation may represents a possible rout for HPV transmission and ultimately reaching and infecting the intrabony odontogenic epithelium.

Currently, surgery is the generally agreed treatment for dentigerous cyst and ameloblastoma. However, it could have a great morbidity and a high recurrence rate especially in ameloblastoma. It seems worth to investigate the effect of different antiviral therapies in managing dentigerous cyst and ameloblastoma, which may finally give the benefit for patients

CONCLUSIONS

The current study confirmed the presence of both HPV–DNA and HPV–L1 capsid protein in ameloblastomas and to a lesser extent in dentigerous cysts. This may indicate a possible relation of HPV with those lesions. Further studies are needed to clarify the exact role of HPV in odontogenic cysts and tumors.

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