Cancer and Oncology Research 1(2): 24-29, 2013
DOI: 10.13189/cor.2013.010202 http://www.hrpub.org

Screening of Ameloblastoma Cases in Ibadan for HPV and *EBV* Genes

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Abstract This study investigated the presence or absence of Epstein-Barr virus and Human papilloma virus genes in ameloblastoma, in order to elucidate the genetic origin of this tumour. Methods and Material: 89 archived formalin-fixed paraffin-embedded specimens of ameloblastoma from the year 2000-2010 were genotyped by polymerase chain reaction for Epstein-Barr virus and Human papilloma virus genes Results: None of the 89 ameloblastoma cases used for this study tested positive for Epstein-Barr virus and Human papilloma virus genes. Conclusions: This study suggested that Epstein-Barr virus and Human papilloma virus genes may likely not be involved in the aetiopathogenesis of ameloblastoma in Ibadan environment. Key Messages: Studies have suggested that HPV might be implicated in the pathogenesis of ameloblastoma. In this study none of the extracted DNA samples from the 89 ameloblastoma cases amplified for HPV primer in the PCR, as this may possibly clear the role of this virus in the etiopathogenesis of ameloblastoma or may indicate need for other primers. Further investigation should be done to clarify EBV role in ameloblastoma.

Keywords Epstein-Barr Virus, Human Papilloma Virus, Ameloblastoma

1. Introduction

Ameloblastomas are an unfathomable group of oral tumors and the most common odontogenic tumor, which occur largely on the basis of the histologic similarities of the tumor and the developing enamel organ. They are named as a result of their resemblance to cells of the enamel-forming organ (1). There is considerable variation in histological patterns, and are classified as follicular, plexiform, acanthomatous, granular cell, basal cell and desmoplastic ameloblastoma (3). Of these, the follicular and plexiform types are the most common (4). Ameloblastomas constitute almost half (48.9%) of the odontogenic tumors with female-to-male and maxilla-to-mandible ratios of 1:1.7 and 1:8 respectively (5). Ameloblastoma is notorious for its recurrences although it is benign in nature.

Ameloblastoma has also been associated with the most complex group of pathogenic viruses. Some authors have hypothesized that Human papilloma virus (HPV) might play a key role also in the pathogenesis of ameloblastoma. However, the results reported in the literature are quite controversial and were not precise (6). Human papilloma viruses (HPV) are members of the papilloma viridae family that infect epithelial cells exclusively. More than 100 types of HPV have been identified, differing in the regulatory sequences and coding potential of their genomes (7), although all types of HPV have the same general organization of the genome (7). Another important virus often associated with various neoplastic developments is the Epstein Barr Virus (EBV) which is a double stranded DNA virus of approximately 175kb in length (8), EBV is a member of the herpesviridae family, gammaherpesviridae subfamily. Although considerable insight has been gained into EBV as an important aetiologic factor in various benign and malignant tumours, virtually little is known about the relationship between EBV and ameloblastoma. A**t** the time of this study, there is no study in Nigeria that have revealed/reported the role played by these viruses (EBV and HPV) or their presence in ameloblastoma.

2. Aim

The aim of this study was to screen for presence or absence of *EBV* and *HPV* genes in ameloblastoma cases in Ibadan

3. Objectives of the Study

To assess the presence or absence of *Epstein-Barr virus* and *Human papilloma virus* genes in ameloblastoma using polymerase chain reaction method and to determine if there is a correlation between EBV and HPV infection and ameloblastoma.

4. Subjects and Methods

4.1. Materials

Eighty-nine (89) blocks of formalin-fixed paraffin-embedded tissues samples diagnosed as ameloblastoma deposited from between the years 2000 and 2010 were retrieved from the archives of the Department of Oral Pathology, College of Medicine of the University of Ibadan, Nigeria. A formalin-fixed paraffin-embedded control (negative) sample of normal salivary gland was selected from the archives of the same institution, as we do not have confirmed tissues with any ofthe viruses as positive sample. The ethical approval for this research was obtained from the UI/UCH joint ethical committee.

4.2. Methods

To de-paraffinized the tissue samples, method of Coura *et al*.,[9] was modified. 5µm sections were cut from each formalin-fixed paraffin-embedded tissue block using a microtome after re-embedded in fresh molten-wax and subsequently placed in 2.5 ml Eppendorf tubes. The sections were then incubated (after adding 2ml of xylene to it in Eppendorf tubes) incubated in a shaking water uniscope SM101 (Surgifriend) bath which is previously set at 55^{o}C for 30 minutes, after which the xylene was carefully pippetted off and the process was repeated two other times. Residual xylene was removed by adding 2ml of absolute ethanol and incubating at 55^{o}C for 5 minutes. After 5 minutes the ethanol was pippetted off and the process was repeated two other times. The residual ethanol was then removed by incubating at 55^{o}C for 30 minutes. The tissue pellets was re-suspended by vortexing.

4.3. DNA Extraction

DNA was extracted from de-paraffinized tissue pellets using 70 µl of digestion buffer made up of 50mM KCl, 1.5mM MgCl2, 10mM Tris-HCl, 0.45% (v/v) Tween-20, and 10mg/ml Proteinase-K (pH 8.3) and incubated in a Stuart orbital incubator S150 at 36oC overnight. After overnight incubation, proteinase-K was in-activated by incubating in a shaking water bath at 95oC for 10 minutes. It was thereafter vortexed and spun in a Hermle-2323 centrifuge at 7000rpm for 5 minutes. Subsequently, the supernatant was collected in autoclaved PCR tubes and stored at -20oC for DNA amplification.

4.4. DNA Amplification

Extracted DNA samples were subjected to polymerase chain reaction amplification. Briefly, the reactions were performed in a final volume of 25µl containing 1.5µl of template DNA, 0.5µl dNTPs, 5µl PCR Buffer, 15.4µl sterile water, 1.5 $MgCl_2$, 0.1 Taq polymerase, 0.5µl of each forward and reverse primers (Integrated DNA Technologies, Coral ville, Iowa, U.S.A). The primers used in the genotyping reactions are shown in table 1. Samples containing the amplification mix were subjected to forty (40) amplification cycles. Conditions for the amplification were with an initial denaturation at 94^{o}C for 5 minutes which was followed by a subsequent denaturation step at 94^{o}C for 60 seconds, an annealing step at 50^{o}C for 60 seconds and initial extension step at 72^{o}C for 60 seconds which was followed by a final extension step at 72^{o}C for 10 minutes**.** 10µl aliquots of polymerase chain reaction amplicon was subjected to electrophoresis on 1.5% agarose gel followed by ethidium bromide staining and viewed on a photo documentation system. The primers used in this study were established EBV (20) and HPV (21, 22, 23) primers, as reported in previous studies.

Table 1. Primers used for the genotyping reactions

Genes	Primer sequence
Barf 1 (EBV) Forward	5′-GGTGAAGCCTCTAACGCTGTCT-3′
Reverse	5′-TCACGGTGCATGTCACAGTAAG-3′
PGMY (HPV) Forward	5′-GCACAGGGACATAACAATGG-3′

5. Results

All the 89 extracted DNA samples from the ameloblastoma cases were amplified for *EBV* and *HPV* genes by polymerase chain reaction (PCR) using *Barf* primer for *Epstein-Barr virus* and *PGMY* primer for *Human papilloma virus* genes.

In all the 89 cases tested by polymerase chain reaction, *Epstein-Barr virus* and *Human papilloma virus* genes of any size did not amplify in any case. Negative control sample (-ve) from normal salivary gland was included, which show no positive amplification for all the genes by polymerase chain reaction. See table 2,3and figure 1-6 for summary.

Table 2 HPV and EBV genotyping features in different histological types of ameloblastoma.

Types of ameloblastoma	Genes in number and percentage (n/%)	
Telangiectatic	*HPV*	*EBV*
Follicular	0/0.0	0/0.0
Plexiform	0/0.0	0/0.0
Acanthomatous	0/0.0	0/0.0
Cystic	0/0.0	0/0.0
Demoplastic	0/0.0	0/0.0
Mixed Plexiform/Follicular	0/0.0	0/0.0
Unspecified	0/0.0	0/0.0

Table 3. HPV and EBV genotyping features in different maxillofacial sites

Types of ameloblastoma	Genes in number and percentage (n/%)	
Mandible	*HPV*	*EBV*
Maxilla	0/0.0	0/0.0
Palate	0/0.0	0/0.0
Buccal cavity	0/0.0	0/0.0
Unspecified	0/0.0	0/0.0

Figure 1. PCR for the *HPV* gene using primer *PGMY* did not yield any amplicons from ameloblastoma cases (lane 1-34) and a negative control sample (normal salivary gland) in –ve lane. The DNA ladder 1kb is in lane M.

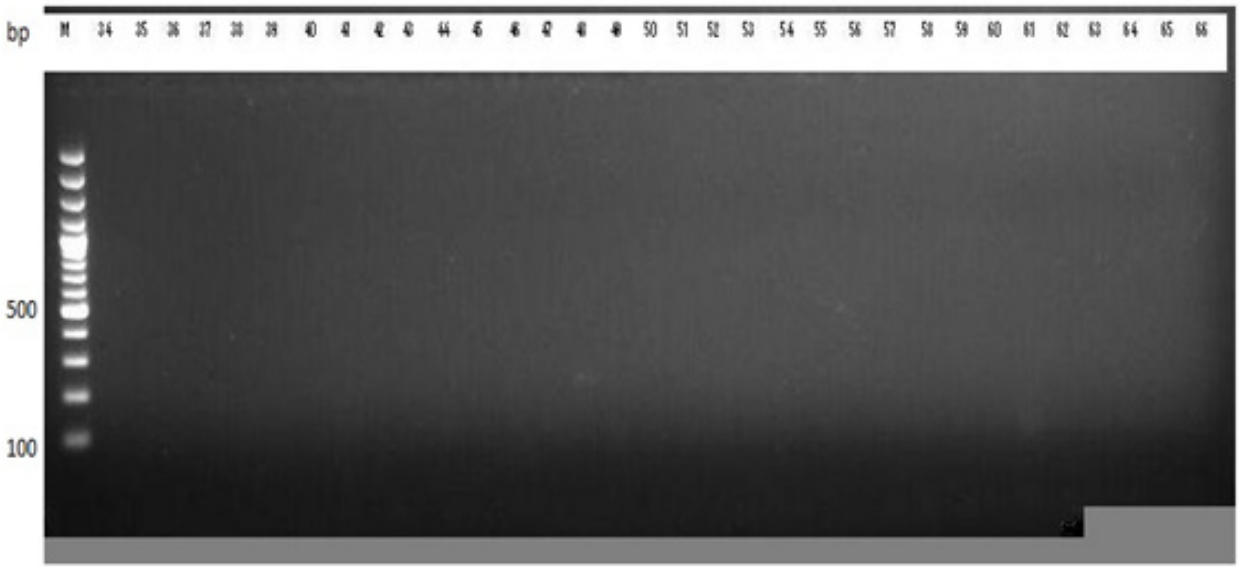

Figure 2. PCR for the *HPV* gene using primer *PGMY* did not yield any amplicons from ameloblastoma cases (lane 34-66). The DNA ladder 1kb is in lane M.

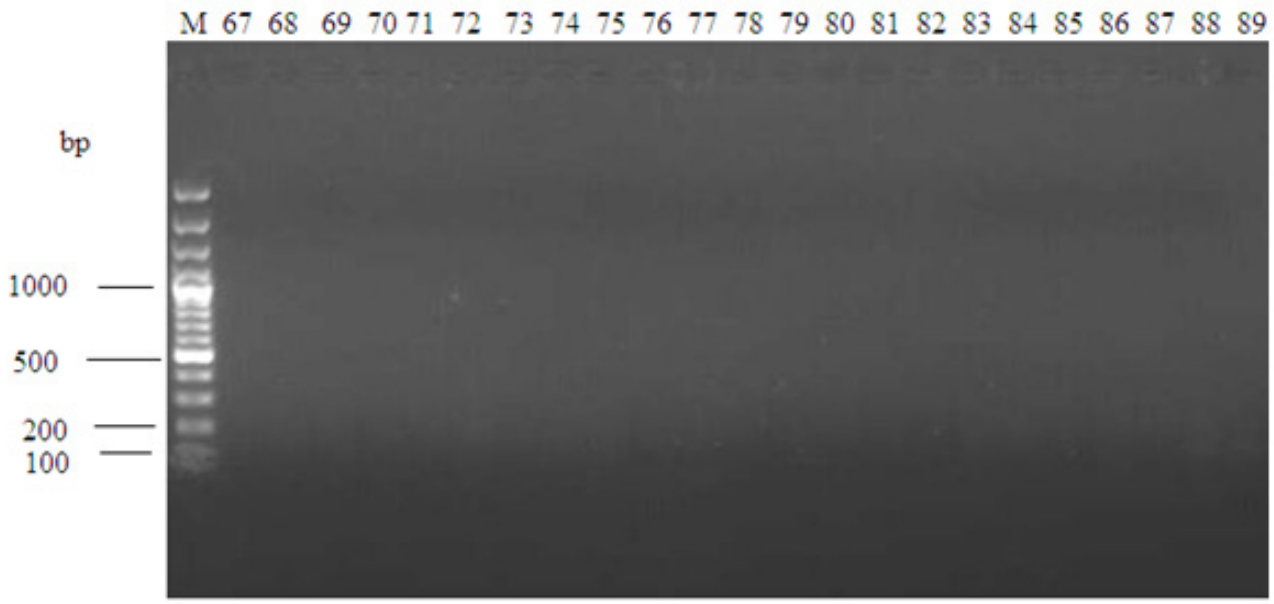

Figure 3. PCR for the *HPV* gene using primer *PGMY* did not yield any amplicons from ameloblastoma cases (lane 67-89). The DNA ladder 1kb is in lane M.

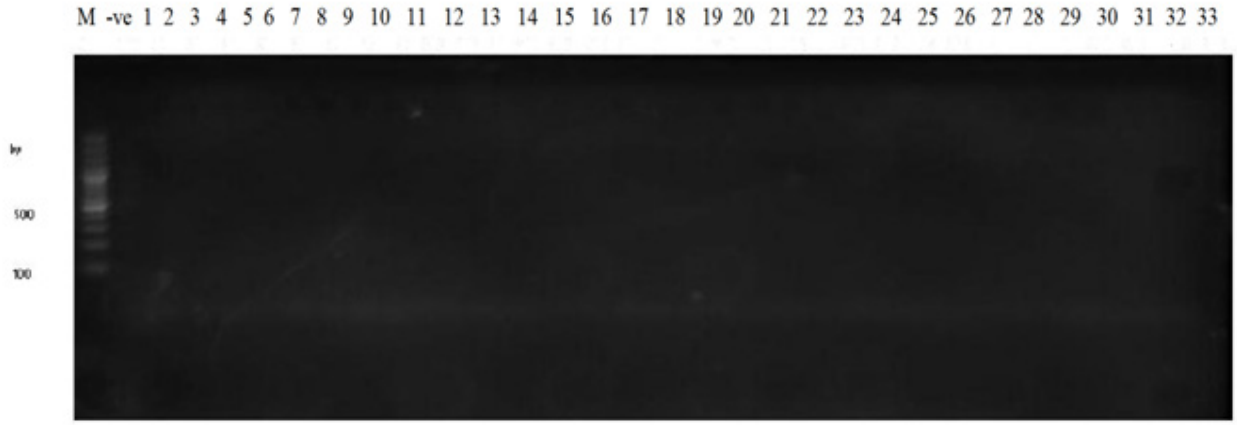

Figure 4. PCR for the *(EBV)* gene using *Barf* primer which did not amplify in ameloblastoma cases (lane 1-33) and a negative control sample (normal salivary gland) in lane -ve. The DNA ladder 1kb is in lane M.

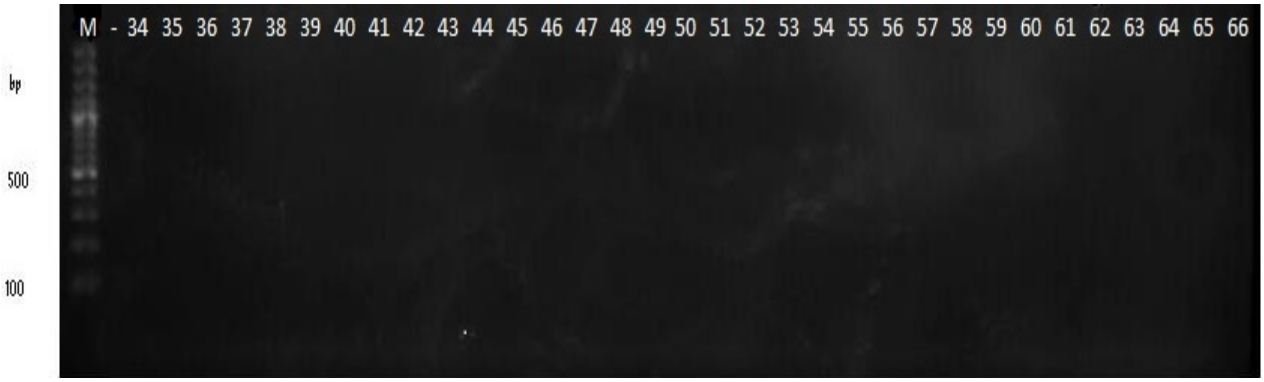

Figure 5. PCR for the *(EBV)* gene using *Barf* primer which did not amplify in ameloblastoma cases (lane 34-66) and a negative control sample (normal salivary gland) in lane (-). The DNA ladder 1kb is in lane M.

Figure 6. PCR for the *(EBV)* gene using *Barf* primer which did not amplify in ameloblastoma cases (lane 34-66) and a negative control sample (normal salivary gland) in lane -ve. The DNA ladder 1kb is in lane M.

6. Discussion

This study attempted to demonstrate the presence of *EBV and HPV* genes in archived formalin-fixed paraffin-embedded ameloblastoma cases by using the polymerase chain reaction method.

None of the extracted DNA samples from ameloblastoma cases amplified for *Barf1 (EBV)* primer in the polymerase chain reaction. This is similar to the immunohistochemical report of Ayoub *et al.,*[2] where only one out of 15 examined benign ameloblastoma showed positive EBV immunoreactions, since all patients included in this study were of Nigerian origin, one cannot completely exclude that EBV might be possibly involved at least in a subset of ameloblastomas occurring in subjects from other geographic areas, as commonly happens in other virus-related neoplastic disorders. The result in this study conflicts with the report of (10). It was suggested that the use of only limited *EBV* genes for the PCR detection might result in a false negative or a false positive detection of oral tumours which likely may be the cause of total negative result in this study (10). Little is known about the relationship between *EBV* genes and oral tumours (10). Moreover, the work done correlating the EBV as a viral aetiology with the occurrence of ameloblastoma is modest in the literature (10; 11).

It has been recently suggested that HPV might somehow be implicated in the pathogenesis of ameloblastoma, the most common odontogenic tumour usually associated with a benign biologic behaviour (6). In this study none of the extracted DNA samples from the 89 ameloblastoma cases amplified for *PGMY (HPV)* primer , as this may likely rule out the possibility of this virus in the etiopathogenesis of ameloblastoma. This result differs from the work of Mokhtari-Azad *et al.,* (12) in Iran, who found that 32% (32/100) of ameloblastomas cases worked on were positive for *HPV-DNA*. This difference probably stemmed from the fact that while this present study used only one type of *HPV* primer, they used fourteen different *HPV* primers that target different regions of the *HPV* genome. Studies have shown that more than one hundred types of HPV have been identified, differing in the regulatory sequences and coding potential of their genomes (7). It has been suggested in some studies (13) that the presence of this virus is only a contamination of the overlying surface squamous epithelium (13), as this was also suggested earlier by Van Heerden *et al.,* (14). Just as this study, results have reasonably rule out any implication of HPV in ameloblastoma (6) while it has been stressed that the presence of HPV-DNA positivity in ameloblastoma likely represented a secondary surgically acquired contamination from the overlying oral mucosa or a secondary event rather than a true infection (14, 15). Discrepancies in study design may also explain the lack of consensus on the role of HPV in oral carcinogenesis (16). HPV is also considered an oral mucosa pathogen, with reported prevalence ranging from 0% to 60% (17, 18, and 19).

7. Conclusion

Although the viral aetiology, either HPV or EBV, of ameloblastoma showed a great contradiction in the literature, it must be noted that a difference in geographic distribution might partially explain these conflicting results as commonly happens in other virus-related disorders, hence, further studies utilizing much more advanced research tools such as in situ hybridization and PCR analysis are highly recommended (2)

Acknowledgement

We thank Mrs. Muinat Fowora of the Molecular and Biotechnology Division and Dr. Iwalokun of Biochemistry Division, Nigerian Institute of Medical Research (NIMR), Lagos for tremendous technical help.

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