CO-PRESENCE OF EPSTEIN-BARR VIRUS AND HUMAN PAPILLOMAVIRUS IS ASSOCIATED WITH A HIGH FREQUENCY OF P53 GENE MUTATION IN CERVICAL CANCER DIAGNOSED IN SOUTHERN NIGERIA

[1,2*]Okoye, J.O. [1]Ngokere, A.A., [1]Onyenekwe, C.C. [3]Eze, U.A. [1]Uduchi, I.O.

[1]Department of Medical Laboratory Science, Nnamdi Azikiwe University, Nnewi, Nigeria

[2]Department of Medical Laboratory Science, Babcock University, Ilishan, Nigeria

[3]Leicester School of Allied Health Sciences, Faculty of Health and Life Sciences, De Montfort University, Leicester LE1 9BH, UK.

***Correspondence Author:** Okoye, J.O.

E-mail: jog.okoye@unizik.edu.ng; +2347031119994

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Abstract

Background: Evidence shows that the co-existence of Human papillomavirus (HPV) and Epstein-Barr virus (EBV) is associated with aggressive and poorly differentiated cervical squamous cell carcinomas (CSCC) phenotype.

Aim: This study aimed to determine the frequency of mutant *p53* (mtp53) gene in single and co-existence of HPV and oncogenic EBV latent membrane protein 1 (E-LMP1) among women diagnosed with CSCC in Southern Nigeria.

Methods: This retrospective cohort study included 105 cervical cancer tissues, diagnosed between January 2016 and December 2018. The tissue sections were immunohistochemically stained for mtp53, E-LMP1, and HPV proteins and scored accordingly. Descriptive, Chi-square test and Pearson's correlation were performed, and statistical significance was set at $p \leq 0.05$.

Result: Among the stained sections, the frequency of HPV, E-LMP1, and E-LMP1/HPV detection was 8.6%, 20.0%, and 42.9%, respectively. The mtp53 detection was higher in E-LMP1/HPV co-presence (86.7%) compared with HPV and ELMP1 mono-presence (55.6%, and 14.3%, respectively), and this was statistically significant ($p < 0.0001$). The prevalence of invasive and non-invasive cases of CSCC were 62.9% and 37.1%, respectively. E-LMP1 and HPV co-presence were significantly higher in invasive cervical cancer (48.5%) compared to non-invasive cervical cancer cases (33.3%) at $p = 0.156$. The E-LMP1 presence was significantly higher in poorly differentiated CSCC (50.0%) compared to the moderately and well-differentiated CSCC (25.0% and 10.5%, respectively) at $p = 0.011$.

Conclusion: This study revealed a higher frequency of EBV/HPV co-infection in CSCC. It also shown- that a higher frequency of mtp53 in E-LMP1 and HPV co-presence, suggesting that E-LMP1 could be responsible for the high CSCC lethality in West Africa.

Keywords: Cervical squamous cell carcinomas, Oncoviruses; Tumours differentiation; Keratinization

INTRODUCTION

Globally, cervical cancer (CC) is the third most common cancer among women with a 56.6% death among newly diagnosed cases (Ferlay *et al*., 2021; Sung *et al*., 2021). In West Africa, it is the second most common cancer and the second cause of cancer-related mortality among women (Ferlay *et al*., 2021). According to the 2020 GLOBACON estimates, West Africa has the world's highest mortality of cervical cancer patients (Sung *et al*., 2021). The high prevalence of the disease in West Africa has been attributed to the persistence of sexually transmitted Human papillomavirus (HPV) as well as low availability and uptake of the HPV vaccine in the region (Centers for Disease Control and Prevention, 2023a).

The reason for the high mortality rate in West Africa is yet to be fully elucidated. However, available evidence shows that keratinizing cervical squamous cell carcinoma of the cervix (CSCC) has higher mortality or adverse outcomes than non-keratinizing SCC (Cooper *et al*., 2015). Cooper and colleagues (2015) also stated that the 5-year disease-specific survival (DSS) was significantly higher for non-keratinizing tumours (63.3%) than for keratinizing tumours (44.8%). Sadly, there is limited published information regarding the keratin status of CC in West Africa. In Southwestern Nigeria, the prevalence of HPV DNA associated with invasive CC ranges from 85.6% to 90.7% (Okolo *et al*., 2010; Orah and Banjo, 2018). Other risk factors for HPV-negative CC are largely unaccounted for due to a lack of published literature. Epstein–Barr virus (EBV) is also sexually transmitted and has also been implicated in CC (CDC, 2023b). According to earlier reports, the pooled prevalence of EBV, detected in CC by its DNA or protein, ranges from 43.6 to 69.6% (Khenchouche *et al*., 2013; Abudoukadeer *et al*., 2015; Aromseree *et al*., 2015; Al-Thawadi *et al*., 2018). Studies show that the co-existence of EBV latent membrane protein 1 (E-LMP1), an oncogenic transmembrane protein, and HPV is associated with aggressive and poorly differentiated SCC phenotype (Al Moustafa *et al*., 2018; de Lima *et al*., 2018). *In vitro* studies also revealed that E-LMP1 induces tumour invasiveness and metastasis by interfering with apoptosis and growth suppression induced by wild-type p53 (Husaini *et al*., 2011; Wang *et al*., 2017). In addition, E-LMP1 has been found to induce up-regulation, phosphorylation, and nuclear localization of mutant p53 (Deng *et al*., 2003; Li *et al*., 2007; Li *et al*., 2008 and Guo *et al*., 2012). To the best of our knowledge, For the first time in West Africa, this study has elucidated the attributable factors to aggressive CSCC by assessing the single and co-existential mutational effects of E-LMP1 and HPV on the p53 gene.

MATERIALS AND METHODS

Study Area

This study was conducted at Babcock University Teaching Hospital and State Hospital Abeokuta, both located in Southwestern Nigeria. The immunohistochemical staining was carried out at the Histopathology Unit, Medical Laboratory Services, Usman Danfodio University Teaching Hospital, Sokoto, Nigeria.

Study population

This retrospective cohort study included cervical tissue biopsies from patients diagnosed with CSCC (n= 105) between 2016 and 2018.

Institutional Review Board Statement

Ethical Consideration and Informed Consent. For this study: ethical approvals were obtained from the State Hospital Abeokuta Ethics Committee (SHA/RES/VOL.2/ 177) and Babcock University Health Research Committee (BUHREC549/18). The study was conducted according to the Declaration of Helsinki by the World Medical Association (WMA) General Assembly.

Haematoxylin and Eosin (H & E) staining

Cut tissue sections were dewaxed in xylene (i)and hydrated by passing through 100%, 90%, 70%, and 50% of alcohol (for 3 minutes each) to distilled water. Then the sections were stained in Erlich's haematoxylin for 15 minutes, washed in running tap water for 2 minutes, differentiated briefly in 1% acid alcohol, washed, and blued in Scott's tap water for 10 minutes. Thereafter, each tissue section was counterstained in 1% alcoholic eosin for 30 seconds and mounted in Dibutylphthalate Polystyrene Xylene (Ochei and Kolhatkar, 2000; Bancroft and Gamble, 2008).

Immunohistochemical technique

Cases of CSCC were subclassified into 3 groups based on keratinization, invasiveness, and extent of differentiation. The presence of viral (E-LMP1 and HPV) antibodies and expression of mutant p53 protein in the tissue sections were determined by immunohistochemical technique as described by Buchwalow and Bocker (2010).

Sections from confirmed positive and negative cases were used as controls. Sections of 3 microns from formalin-fixed paraffin-embedded tissue samples were mounted on charged slides and air-dried for 2 hours at 60ºC. Sections were deparaffinized in three changes of xylene and hydrated through grades of alcohol. Thereafter, tissue sections were subjected to heat epitope retrieval using citrate buffer at pH 6.0 at 95ºC. The retrieval solution was heated in a water bath to 65ºC before the slides were introduced and heated to 95ºC. The sections and buffer were further heated at 95ºC for 20 mins. Sections were then allowed to cool at room temperature for 20 mins and adequately washed using phosphate-buffered saline (PBS) at pH 7.4. This was followed by placing the tissue sections in a peroxidase blocker, allowing them to stand for 5 minutes, and subsequently washing them using PBS. The circumferences of tissue sections on slides were marked round with a grease pencil and subsequently covered with primary antibodies (diluted with immunodetection protein blocker/antibody diluent), allowed to stand for 60 mins, and adequately washed using PBS. Tissue sections further were covered with a biotin link, allowed to stand for 10 minutes, and washed using PBS. Subsequently, tissue sections were covered with a horseradish peroxidase label, incubated for 10 mins, and washed with deionized water. Tissue sections were covered with DAB substrate-chromogen solution (one drop of DAB chromogen in one ml of immunodetection DAB buffer), allowed to stand for 5 mins, and rinsed with deionized water. The sections were counterstained with haematoxylin for 2 mins, rinsed in PBS and water, dehydrated, dealcoholized in xylene, and permanently mounted using coverslips. Photomicrographs were taken for documentation. Immunoglobin G1-based primary antibodies included ELMP1 and HPV16/18 (from Bio SB Inc USA, Ref: BSB 5487-CSI-4 and BSB 5655-SB24, respectively). The primary p53 antibody was purchased from Bioss Inc., USA, bs-0913R; Swiss Prot P04637). E-LMP1, HPV, and mtp53 staining (brown) in the tissues was scored using a scale of 0, +1, +2, and +3 based on intensity. Scores 0 and +1 were considered negative while scores +2 and +3 were considered positive (Okoye *et al*., 2015).

Statistical analysis

Data were analyzed using GraphPad Prism version 6 (GraphPad Software, Inc., California, USA). The Chi-square/Fisher exact test was used to evaluate the association between variables (keratinization, invasion, differentiation, and mtp53 expression) and viral infections. Pearson's correlation was used to find the relationship between variables and viral infections. Analysis of variance (ANOVA) was performed to ascertain the differences between the ages of the variables. A p-value of <0.05 was considered as statistically significant at a 95% confidence interval.

RESULTS

The median age of participants with CSCC was 54 years, respectively. The frequency of HPV, E-LMP1, and E-LMP1/HPV detection was 8.6%, 20.0%, and 42.9%, respectively (**Figure 1**). Similarly, the frequency of mtp53 protein was higher in E-LMP1/HPV co-existence (86.7%) compared to HPV, and ELMP1 mono-existence (55.6%, and 14.3%, respectively) at $p< 0.0001$. The frequency of E-LMP1/HPV detection was significantly higher in invasive cervical cancer compared with non-invasive cervical cancer cases ($p= 0.156$) while the prevalence of HPV mono-infection was higher in the latter than the former ($p= 0.012$). The prevalence of E-LMP1 protein was higher in invasive and poorly differentiated CSCC compared with moderately and well-differentiated SCC ($p= 0.627$ and 0.011, respectively). The frequency of E-LMP1 protein was higher in non-keratinizing CSCC than in keratinizing CSCC ($p= 0.067$) while the frequency of E-LMP1/HPV was higher in keratinizing SCC compared with non-keratinizing CSCC ($p= 0.674$). HPV mono-infection was significantly associated with keratinization ($p= 0.030$).

An increasing frequency of E-LMP1 expression was observed with tumour dedifferentiation when compared with other viruses (**Table 1**). An insignificant positive correlation was observed between HPV infection and E-LMP1 expression (r= 0.292, p= 0.094). A significant relationship was observed between ELMP1/HPV detection and mtp53 expression (r= 0.739, p< 0.001).

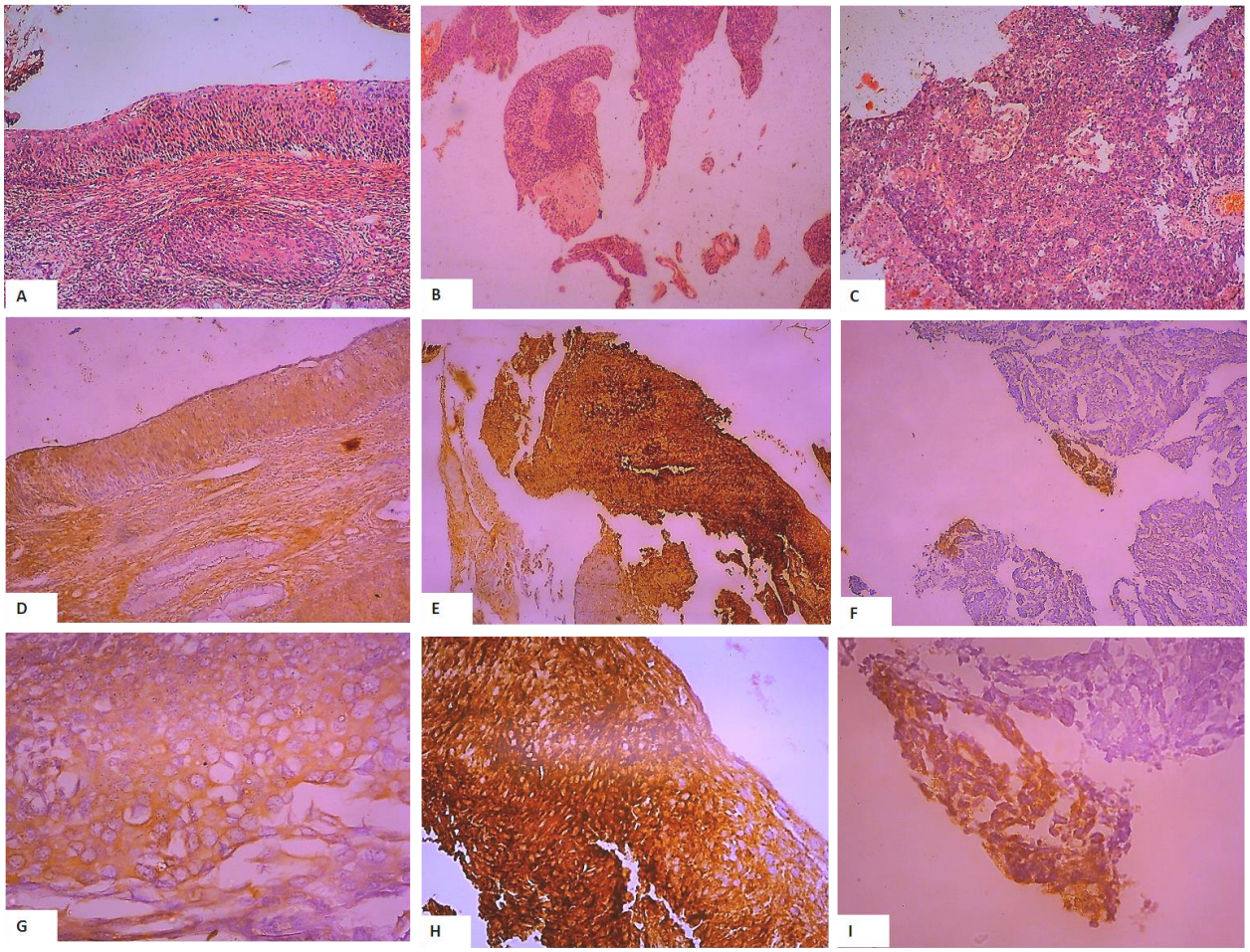

Figure 1: Section of the cervix stained by H&E (A-B; x100 magnifications) and immunohistochemistry (D-F; x100 magnification, G-I; x400 magnification)

Figures 1B and 1C show moderate positive staining intensity for ELMP1 protein. Figures 1E and IH show high positive staining intensity for HPV protein while figures 1F and 1I show focal staining for mutant p53 protein.

Table 1: Frequency comparison of HPV and E-LMP1 detection in cervical cancer grades

Variables	Age (years)	No. (%)	HPV Alone	LMP1 Alone	LMP1/ HPV	X^2	p-value
Invasive CSCC	51.92±8.95	66 (62.9)	2 (3.0)	12 (18.2)	32 (48.5)	7.775	0.021
Non-invasive CSCC	60.80±15.87	39 (37.1)	7 (17.9)	9 (23.1)	13 (33.3)		
Keratinizing CSCC	60.00±11.56	34 (32.4)	4 (11.8)	3 (8.8)	16 (47.1)	3.960	0.138
Non-Keratinizing CSCC	51.55±11.05	71 (67.6)	5 (7.0)	18 (25.4)	29 (40.8)		
Well-diff. CSCC	45.50±13.44	57 (54.3)	7 (12.3)	6 (10.5)	23 (40.4)	7.870	0.096
Moderately diff. CSCC	47.50±11.24	36 (34.3)	2 (5.6)	9 (25.0)	16 (44.4)		
Poorly diff. CSCC	59.00±8.89	12 (11.4)	0 (0.0)	6 (50.0)	6 (50.0)		
Total	53.32±12.54	**105 (100)**	**9 (8.6)**	**21 (20.0)**	**45 (42.9)**		

Keys: SCC= Squamous cell carcinoma, diff.= differentiation Statistics: Chi-square/Fisher test

DISCUSSION

In West Africa, there is a paucity of studies on the role of E-LMP1 and HPV interaction in SCC emergence. To assess their roles in this highly lethal disease among West African women, this study aimed to evaluate the frequency of mtp53 in single and co-presence of E-LMP1 and HPV in invasive, keratinized, and poorly differentiated CSCC. The frequency of HPV associated with CSCC in this study is lower than the frequencies earlier reported in Southwestern Nigeria; 85.6 to 90.7%) (Okolo *et al*., 2010; and Orah and Banjo, 2018). The reason for the variation in HPV frequency could be due to the difference in the technique used for HPV detection; IHC versus polymerase chain reaction. The frequency of keratinized CSCC in this study is also lower than the frequency of 46.2% reported by Cooper *et al.* (2015). This study shows a higher frequency of HPV-associated keratinized CSCC (58.9%) than HPV-associated non-keratinized CSCC (47.8%). This is at variance with the findings of Cooper *et al*. (2015) who reported a higher frequency of HPV in non-keratinized oropharyngeal SCC (OSCC; 64.3%) than in keratinized SCC (40.6%). Other studies also reported higher HPV detection in non-keratinized OSCC (69% and 80.8%) than in keratinized oro-pharyngeal SCC (8% and 20.7%) (Chernock *et al*., 2009; Chernock *et al*., 2012). This suggests that the activity of HPV in keratinized CSCC is higher than that of the OSCC. The reason for this deviation is unknown. Cooper *et al*. (2015) reported a significantly higher survival rate among women with non-keratinizing OSCC than those with keratinizing SCC. Further studies are required to ascertain whether the latter findings in OSCC apply to CSCC.

Interestingly, the frequency of E-LMP1 in this study (62.9%) is higher than the frequency reported in Algeria (29%) by Khenchouche *et al*. (2013), even though both studies used the same technique. In this study, there was a higher frequency of E-LMP1-associated non-keratinized CSCC (66.2%) than E-LMP1-associated CSCC (55.9%). This suggests that EBV infection could be a major player in transforming cervical epithelia, especially among HPV-negative women in West Africa. The frequency of EBV/HPV co-infection in this study (42.9%) is higher than the frequency reported in Thailand (30%) and Syria (34%) by Aromseree *et al*. (2015) and Al-Thawadi *et al*. (2018), respectively. Al-Thawadi *et al*. (2018) also observed a strong relationship between E-LMP1/HPV and poorly differentiated SCC compared with a single infection with HPV or EBV. Additionally, this study revealed a direct relationship between E-LMP1, tumour invasiveness, and poorly differentiated CSCC. This agrees with the findings of earlier studies (Al Moustafa *et al*., 2018; de Lima *et al*., 2018) and suggests that E-LMP1 could be a major player in the emergence of poorly differentiated CSCC. The high frequency of mtp53 in E-LMP1 and HPV co-presence suggests that co-infection with HPV and EBV favours the increased expression of HPV E6, resulting in p53 mutation, ultimately leading to CSCC development. This study included a small sample size which may limit its conclusion. However, the study could serve as a baseline for future studies involving a large sample size. More studies are also required to identify the mechanism behind HPV-negative EBV-positive CSCC.

CONCLUSION

This study revealed that E-LMP1 increases the risk of p53 mutation in HPV-positive cervical lesions. This study suggests that E-LMP1 could be responsible for the aggressive CSCC in West Africa, especially among HPV-negative women.

RECOMMENDATIONS

An improved screening policy should be developed and implemented for sexually active populations to ensure early detection and management of infected individuals.

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