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ABSTRACT

Background: **Persistent infection with specific oncogenic types of human papilloma virus (HPV) has been strongly implicated in the aetiopathogenesis of cervical carcinoma by several epidemiological, clinical and molecular studies. Studies have also shown that differences exist in the prevalence and distribution of HPV genotypes among different histological types of cervical carcinoma. The objectives of this study were to determine the HPV prevalence and distribution of HPV genotypes in correlation with diverse histological subtypes of cervical cancer in Maiduguri, Nigeria.**

Materials and Methods: **This was a descriptive and retrospective study. Sixty-three archived paraffin-embedded tissue blocks and slides with confirmed diagnoses of cervical cancer during the study period (2013-2015) were retrieved. They were reviewed and classified according to World Health Organization (WHO) classification. The laboratory procedures included deparaffinization of tissue samples, DNA extraction, Polymerase Chain Reaction (PCR), gel electrophoresis and HPV genotyping by reverse hybridization line probe assay.**

Results: **Among the samples analysed, the proportion of squamous cell carcinoma was 84.1%, while adenocarcinoma and adenosquamous carcinoma accounted for 12.7% and 3.2% respectively. The overall prevalence of HPV-specific DNA in biopsies of cervical carcinoma was 69.8%. Multiple HPV types were found in 61.4% of the cases, while single HPV infection accounted for 38.6% of the total cases. The HPV-DNA positivity in squamous cell carcinoma, adenocarcinoma and adenosquamous carcinoma was 71.7%, 50.0% and 100% respectively. HPV18 was more predominantly associated with adenocarcinoma**

than with squamous cell carcinoma. Together, HPV16 and HPV18 accounted for 59.4% of HPV-positive cervical carcinomas.

Conclusion: **HPV-DNA was found in majority of the examined cervical adenocarcinomas and adenosquamous carcinoma, similar to that of squamous cell carcinoma. Although the rare subtypes of adenocarcinoma were not associated with HPV infection, HPV-based tests and vaccines would significantly prevent cervical cancer.**

INTRODUCTION

Cervical cancer is the second most common cancer in women worldwide, after breast cancer.[1] It was estimated that there were 530,000 cases of cervical cancer and 270,000 deaths annually.[1]The burden of cervical cancer is quite high with an estimated 80% of cases occurring in the developing countries.[2] Sub-Sahara Africa is the region with the highest incidence of cervical cancer in the world with concomitant high mortality.[2]

In Nigeria, cervical cancer is currently the second most common cancer in women after breast cancer with about 14,089 new cases diagnosed annually and 8240 deaths.[3]Most of the studies from different parts of Nigeria have also indicated that cervical cancer is the commonest gynaecological cancer. In Maiduguri, Northeastern Nigeria, it was the commonest gynaecological malignancy representing 70.5% of the total gynaecological cancers.[4]

Studies have shown that squamous cell carcinoma accounts for 70-80% of invasive carcinomas, while adenocarcinoma and adenosquamous carcinomas comprise 10-15% of all cases.[5] Persistent high-risk HPV infection is a well-established causative factor and responsible for 87-100% of squamous cell carcinoma and 65-85% of adenocarcinoma cases.[6,7] In squamous cell tumours, HPV-16 predominates, but HPV-18 is relatively more predominant in adenocarcinoma and adenosquamous tumours.[8] In one study of HPV genotypes in adenocarcinoma subtypes, the usual type adenocarcinoma accounted for 83.1%of cases, while rare histological variants accounted for a few percent of cases individually.[7]

MATERIALS AND METHODS

Case Selection and Histological Subtyping: The present study was conducted at the University of Maiduguri Teaching Hospital (UMTH) which is the apex referral centre for persons and specimens in the Northeastern region of Nigeria.

Consecutive cases of 63 confirmed cervical carcinomas were retrieved from the archives of the Department of Histopathology of the UMTH between January 2013 and December 2015.Clinicopathological parameters were obtained from the histopathology request cards. The haematoxylin and eosin (H&E) stained-slides were reviewed and classified according to WHO histologic classification. This research work was carried out at the Biotechnology Centre, University of Maiduguri and Department of Histopathology, University of Maiduguri Teaching Hospital, Maiduguri. The study protocol was approved by the Research and Ethics Committee of the Hospital.

DNA Extraction: DNA was extracted from 8-10 micron sections of FFPE tissues using *QIAamp*® DNA FFPE Tissue kit from Qiagen (Hamburg, Germany) according to manufacturer's instruction. The protocol was

slightly modified to allow overnight digestion with *Proteinase K*.

Following extraction, DNA was quantified using *NanoDrop2000C* spectrophotometer (Thermos Scientific, USA). Concentration was determined based on absorbance at 260nm.

HPV-DNA Detection and Genotyping: PCR was run for human Hb *beta* subunit (β-globin) to ascertain the quality of the extracted genomic DNA and the viability of the tissue for PCR detection of HPV. A primer set which targets a 122-bp sequence of β-globin was used (*Inqaba Biotech West Africa)*;

Forward:
5′CTTCTGACACAACTGTGTTCACTAGC 3′
Reverse:
5′TCACCACAACTTCATCCACGTTCACC 3′.

Broad-spectrum HPV-DNA amplification was performed on the β-globin-positive DNA samples using the short PCR fragment (SPF10) primer set. The SPF10 consensus primers amplify a 65-bp fragment from the L1 region of the HPV genome.

HPV forward: 5'-
GCiCAGGGiCACAATAATGG-3'
HPV reverse: 5'-
GTiGTATCiACAACAGTAACAAA-3′.

The PCR amplification for HPV genotyping using the INNO-LiPA HPV genotyping Extra II Amp was carried out according to the manufacturer's recommendation. Reaction was carried out in 40 μl using the provided Master mix containing biotinylated primers in buffers with dNTPs/dNTP-mix, $MgCl_2$, AmpTaq Gold 360 DNA polymerase, uracil N-glucosidase and 0.05% NaN_2 as preservative. A 10μl of HPV positive samples as detected previously was added to form a final volume of 50μL. Positive PCR control as provided in reaction kit contains HPV6 DNA and HLA-DPB DNA and 0.05 NaN_2 as preservative.

The following PCR condition was used as described by the manufacturer; 37^0C,10min; 94^0C, 9 min [94^0C, 30sec; 52^0C,45^0sec; 72^0C,45sec] x40

After DNA amplification, HPV genotype was determined by a reverse line probe assay for the identification of 28 different HPV genotypes.

A 10 μl of the purified PCR product was denatured and hybridised to genotype-specific oligonucleotide probes immobilised as parallel lines on a nitrocellulose membrane strips, following the manufacturer's instructions (INNO-LiPA HPV genotyping kit, Innogenetics, Ghent, Belgium). The 28 probes for 25 different HPV genotypes in each INNO-LiPA strip are for 18 high-risk (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82) and 7 low-risk (6, 11, 40, 43, 44, 54, 70) HPV types. A positive hybridization and color reaction gives a purple precipitate in form of parallel lines. The results of hybridization were assessed visually; the conjugate control line on the strip was aligned to the corresponding line on the interpretation sheet, and the result of HPV genotype was read accordingly.

Statistical Data Analysis: The information on the cases of cervical cancer under review from the histopathology request cards and the results from the study were entered into a computer program. Data analysis was carried out using the statistical package for social sciences (SPSS™) version 20.0 Chicago IL, USA, computer software. Statistical significance was assessed using the student's t-test. Correlation was evaluated using Pearson's correlation test. P value < 0.05 was considered statistically significant. The relationship between the detected HPV genotypes and histological subtypes of cervical carcinoma was presented in table.

RESULTS

During the study period between January 2013 and December 2015, a total of 602 cases were diagnosed as cancer in the Department of Histopathology of the University of Maiduguri Teaching Hospital, Maiduguri. A total of 105 (17.4%) cancer cases were of female genital tract malignancies, out of which 82 (78.1%) cases were diagnosed as cervical cancer. Sixty-three (63) cases fulfilled the inclusion criteria and were subjected to genomic DNA extraction and HPV-DNA detection by PCR. There were 53 cases (84.1%) of squamous cell carcinoma, 8 cases (12.7%) of adenocarcinoma and 2 cases (3.2%) of adenosquamous carcinoma. In adenocarcinoma subtypes, there were 4 cases (50.0%) of the usual-type adenocarcinoma and 2 cases (25.0%) of villoglandular, while endometrioid and clear cell adenocarcinoma each accounted for 12.5% of the total adenocarcinoma cases. HPV-specific DNA was detected in 44 of the 63 analysed samples, which was a prevalence of 69.8%.

Among the samples with a positive HPV-DNA status, ten (10) different high-risk HPV genotypes were detected, and in order of decreasing frequency, they included HPV16 (39.6%), HPV18 (19.8%), HPV45 (12.9%), HPV52 (8.9%), HPV51 (5.0%), HPV59 (2.9%), HPV35 (1.9%), HPV31 (1.0%), HPV58 (1.0%), and HPV73 (1.0%). A co-infection with low-risk HPV11 was also observed in 6 (5.9%) of the samples. Both single and multiple high-risk HPV infections were observed. There were 17 (38.6%) cases of single HPV infection and 27 (61.4%) cases of multiple HPV infections.

Relationship between Histological Types of Cervical Carcinoma and HPV Genotypes: Of the 44 HPV DNA-positive samples, 38 (86.4%) cases were found to be squamous cell carcinoma, while 4 (9.1%) and 2 (4.5%) cases were adenocarcinoma and adenosquamous carcinoma respectively. The proportion of HPV-specific DNA positivity indicated that 71.7% (38 of 53) of squamous cell carcinoma had HPV DNA. Similarly, 50.0% (4 of 8) of adenocarcinoma, and 100% (2 of 2) of adenosquamous carcinoma had HPV DNA.

All of the ten different high-risk HPVs and the low-risk HPV11 were observed in squamous cell carcinoma as either single or multiple-type HPV infection. Single and multiple-type HPV infections accounted for 39.5% and 60.5% respectively. Only HPV16 and HPV18 were identified as single HPV infection in cases of squamous cell carcinoma, while the other HPVs occurred as multiple-type HPV infection (Table 1). HPV16 was the commonest viral type detected in 89.5% of the HPV-positive squamous cell carcinomas. These included cases in which HPV16 was identified as a single HPV infection (31.6%) and cases with multiple-type HPV infection (57.9%). HPV18 was the second most common viral type after HPV16 and was detected in 42.1% of all HPV-positive squamous cell carcinomas; as a sole HPV type in 7.9% and with multiple-type HPV infection in 34.3%. The remaining HPVs were detected in multiple-type HPV infection. In adenocarcinoma cases, seven HPVs were identified and they included HPV16, HPV18, HPV45, HPV35, HPV52, HPV58 and HPV11. The single HPV infection was detected in 25% of cases while multiple-type HPV infection was found in 75% of adenocarcinomas. HPV16 was the commonest viral type detected in 100% of the HPV-positive adenocarcinomas, which included 25.0% as a single HPV infection in the usual-type adenocarcinoma and with multiple-type HPV infection in 75.0% of cases involving the usual-type and villoglandular

adenocarcinoma as shown in Table 1. HPV18 was also the second most common viral type detected in HPV-positive adenocarcinomas. It was identified as multiple-type HPV infection and detected in 3 of the 4 cases (75%) of adenocarcinoma comprising 2 usual-type and 1 villoglandular adenocarcinomas. HPV45, HPV52 and HPV58 were detected in 25% of cases of adenocarcinoma of usual-type category. HPV35 and HPV11 were identified in the villoglandular adenocarcinoma. In the adenosquamous carcinoma category, both single and multiple-type HPV infections were observed in 50% of cases each. HPV16 was observed in 100% of HPV-positive adenosquamous carcinoma –as a single HPV type in 50% and with HPV18 in multiple-type HPV infection in 50% of cases as shown Table 1. Overall, in all the three main categories of histological diagnosis, HPV16 was detected in 89.5% of the 38 cases of squamous cell carcinoma, and 100% of both adenocarcinoma and adenosquamous carcinoma. Similarly, in all the three categories of histological diagnosis, HPV18 was detected in 42.1% of the 38 cases of squamous cell carcinoma, in 75.0% of the 4 cases of adenocarcinoma and in 50.0% of the 2 cases of adenosquamous carcinoma. Although it was not statistically significant, HPV18 was associated more with adenocarcinoma than with squamous cell carcinoma.

Table 1

Relationship between HPV Genotype and Histological Type

HPV Genotype	**Histological Type**			**Total**
	SCC	**AD**	**ADS**	
HPV16	12	1(U-AD)	1	14
HPV18	3	0	0	3
HPV16 & 18	1	0	1	2
HPV16 & 45	1	0	0	1
HPV16 & 52	4	0	0	4
HPV16 & 11	1	0	0	1
HPV45 & 73	1	0	0	1
HPV16,18 & 45	2	0	0	2
HPV16,18 & 52	2	1(U-AD)	0	3
HPV16,18 & 35	1	0	0	1
HPV16,18 & 11	1	0	0	1
HPV16,45 & 51	1	0	0	1
HPV16,31 & 52	1	0	0	1
HPV16,45 & 11	1	0	0	1
HPV16,18,45 & 51	2	0	0	2
HPV16,18,35 & 11	0	1 (V-AD)	0	1
HPV16,18,45 & 58	0	1(U-AD)	0	1
HPV16,18,45,51 & 52	1	0	0	1
HPV16,18,45,59 & 11	2	0	0	2
HPV16,18,45,51 & 59	1	0	0	1
Total	**38**	**4**	**2**	**44**

Legend
HPV: Human papillomavirus
SCC: Squamous cell carcinoma
AD: Adenocarcinoma
ADS: Adenosquamous carcinoma
U-AD: Usual-type adenocarcinoma
V-AD: Villoglandular adenocarcinoma

DISCUSSION

The oncogenic role of high-risk human papillomavirus in the development of cervical carcinoma is well-established as reported by several studies worldwide.[1,2,6,7-11] Although previous studies reported low prevalence of HPV in adenocarcinoma relative to that of squamous cell carcinoma, more recent reports have shown a higher prevalence of HPV in adenocarcinoma following histopathological review and subclassification of adenocarcinoma.[7,12] More sensitive techniques have also been shown to identify higher prevalence of HPV in adenocarcinoma.[13]

In this study, the overall prevalence of HPV was 69.8%. Similar findings were reported in Serbia[14] and Ethiopia.[15] According to different studies, the prevalence of HPV in cervical cancer varies widely between 79-100%,[1] due to geographical variation,[16] sample origin,[1] methods of DNA extraction,[17-21] and sensitivity and specificity of HPV detection methods.[19,20] The five most prevalent high-risk HPV genotypes in this study in decreasing order of frequency were HPV16, HPV18, HPV45, HPV52 and HPV51. This finding is a global phenomenon most especially for the first three HPVs as reported in several studies across the world.[9-11,14] HPV16 and HPV18 were the most common HPVs detected in this study and accounted for 39.6% and 19.8% respectively, and 59.4% combined. These two high-risk HPVs are the most frequently studied and consequently implicated in the causation of cervical cancer worldwide.[9, 11, 12, 22-25]

The HPV prevalence in the various histological types of cervical cancer was 71.7%, 50.0% and 100% for squamous cell carcinoma, adenocarcinoma and adenosquamous carcinoma respectively. Such observations were also made by some other studies across the globe.[23-25] Similar to the overall HPV prevalence in this study, the 5 most common high-risk HPVs identified in squamous cell carcinoma in decreasing order of frequency were HPV16, HPV18, HPV45, HPV52 and HPV51. Together, they accounted for 85% of HPVs in squamous cell carcinoma. The predominance of HPV16 and HPV18 in squamous cell carcinoma is worldwide and has been observed in several studies.[8,11,20,25] However, studies from Ghana and Republic of Benin have reported respectively that HPV18 and HPV39 and not HPV16, were the predominant HPVs in squamous cell carcinoma and suggested a geographical variation for the observed difference. [16, 26]

The relatively lower rate of HPV-DNA positivity detected in adenocarcinoma as compared to that of squamous cell carcinoma is partly due to low viral load in glandular epithelium.[7,12] The glandular epithelium is characterised by the presence of only the integrated HPV-DNA in the infected cells. In contrast, the infected squamous cells contain both the episomal and integrated HPV-DNA.[7, 12]

In decreasing order of frequency, the 5 most common HPVs detected in adenocarcinoma in this study were HPV18, HPV16, HPV35, HPV45 and HPV11. An interesting finding in this study was that a fewer number of HPVs were observed in adenocarcinoma when compared to squamous cell carcinoma (7 Vs 11). Similar finding of few HPVs in adenocarcinoma was reported by Pirog et al.[7] In the various adenocarcinoma subtypes in our study, HPV-DNA was detected in 75.0% of the usual-type adenocarcinoma, in 25.0% of villoglandular and none in endometrioid and clear cell carcinoma. Both the usual-type and villoglandular adenocarcinomas were predominantly associated with HPV16 and HPV18. In a study by Pirog et al,[7] HPV DNA was also detected in 71.8% of classic (usual-type) adenocarcinoma. Although the rare subtypes of adenocarcinoma are mainly not associated with HPV, villoglandular adenocarcinoma is predominantly associated with HPV16 and HPV18.[13,27] An 100% HPV DNA positivity in villoglandular adenocarcinoma was reported by H J An et al.[13] The observation of HPV-negativity in clear cell carcinoma in this study was similar to a report by Pirog et al.[12] Although there was no HPV DNA detected in endometrioid-type adenocarcinoma in this study, two different studies by Pirog et al[7,12] reported 27.3% and 100% HPV DNA positivity in their cases of endometrioid-type adenocarcinoma.

Adenosquamous carcinoma accounts for 5-25% of all cervical carcinoma and is found to be highly associated with HPV infection.[12] In this study, adenosquamous carcinoma accounted for 3.2% of all cases of cervical carcinoma, and HPV DNA was identified in 100% of the cases comprising HPV16 and HPV18. Similar findings of high prevalence of HPV in adenosquamous carcinoma and the predominance of HPV16 and HPV18 were reported by some other studies.[12,13]

Overall, HPV18 was comparatively associated more with adenocarcinoma (in 75% of cases) than with squamous cell carcinoma (in 42.1% of cases), although this association was not statistically significant. Several studies have observed that there is relationship between HPV genotype distribution among different histological types of cervical carcinoma with higher rate of HPV18 in adenocarcinoma than in squamous cell carcinoma.[8, 11, 14, 25]

CONCLUSION

The results of this study have shown that high-risk human papillomaviruses especially HPV16 and HPV18 were associated with all the various histological types of cervical carcinoma. It implies that the currently available HPV vaccines and HPV DNA-based screening tests could significantly prevent occurrence of cervical cancer in Maiduguri, Nigeria.

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