Herpes Simplex Virus type 2, Epstein Barr Virus and Human Papilloma Virus Co-infection in Cervical Squamous Cell Carcinoma among Sudanese Females

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Abstract

Introduction: Squamous cell carcinoma (SCC) of the uterine cervix is the second cancer among Sudanese females, peculiar tendency to present late due to lack of education and efficient screening programs. High risk HPV 16, 18 have been detected, also existence of HPV 35 was documented. There are postulations to incriminate other viruses in the etiology of cervical carcinoma like Epstein-Barr virus and herpes simplex virus the exact role of these viruses in the etiology of cervical cancer is not clear. The aim of this study was to clarify the existence and prevalence of these viruses among Sudanese women with cervical cancer, and verify the existence of new players in cervical cancer.

Method: This was observational case control study conducted in Khartoum state-Sudan during period from October 2013 to June 2016. One hundred and eighty paraffin-embedded tissue blocks were included in this study; 98 blocks from patients with cervical squamous cell carcinoma (case group) versus 82 cervix tissue blocks malignancy-free (control group), were subjected to detection of HSV-2, EBV and HPV infections using Polymerase Chain Reaction (PCR) and Immunohistochemistry (IHC) techniques.
Results: HPV infection was detected in 41.8% among cases, while the healthy individuals reported 2.4% with high statistically significant different, the P value was 0.000 with 28.8 odds ratio (Ors 28.8; 95% CIs=6.686-123.86; P=0.000), ladies with HPV infection have 28.8 chance to develop cervical cancer. HSV-2 revealed positivity in 6.1% of the cases biopsies, and in 1.2% of the controls biopsies, with no statistically significant correlation, the P value was 0.128. EBV was positive in 2% of the cancer samples, while the non cancer samples reported in 0.00%, with no statistically significant correlation, the P value was 0.501.

Conclusion: HPV, HSV-2 and EBV infections were present in cervix SCC samples, but with different positive rates. HPV remains the major virus associated with cervix SCC tumorigenesis. Both HSV-2 and EBV their roles in the development of cervical SCC appear as bystanders rather than oncoviruses or cofactors. Further studies are needed to determine its precise role.

Keywords: HPV, HSV, EBV, cervix, Cervical cancer, Co-infection, Sudan.

Introduction

Cervical cancer is a cancer that develop from the cervix. 1 It is due to the abnormal growth of cells that have the capability to invade or spread to other parts of the body. 2 Cervical cancer typically develops from precancerous lesion over 10 to 20 year. 3 Worldwide, cervical cancer is both the fourth-most common cause of cancer and the fourth-most common cause of death from cancer in women. 4 Cervical cancers affect women in the prime of their lives, despite being one of the few cancers that can be prevented with simple testing. 5 Cervical cancers yet still the commonest cause of cancer death among women in poor countries. 6 The causative association between Human Papilloma Virus (HPV) and Squamous Cell Carcinoma (SCC) was recognized. 7 Persistent infections by oncogenic types of HPV are considered the etiological factor for cervical carcinoma development. Although HPV is not a sufficient factor for developing cervical cancer, several other co-factors were identified, such as: infection by other sexually transmitted diseases (STI) (HIV, Chlamydia trachomatis, Cytomegalovirus 8 and Herpes Simplex Virus (HSV-2)). 9 Sexually transmitted agents, particularly viral infections, have been postulated to have a synergistic role in the carcinogenesis of cervical neoplasia. 10 There is evidence that the uterine cervix is the site of shedding of the HSV-2 and EBV. Correlation of HSV-2 and EBV infections with the development of cervical lesions/cancer is still the main question nowadays. It is still doubtful what the prevalence of these viruses in cervix is and if HSV-2 and EBV can act as co-factors in cervical carcinogenesis.

Materials and Methods

Samples:

In this observational case control study, the study population consisted of 98 formalin fixed paraffin embedded (FFPE) cervical tissues diagnosed with SCC and 82 FFPE cervix tissues malignancies free.

Sample processing

One section from each block measures four micrometers was cut and then stained in H&E to confirm diagnosis of each block. Then four sections were cut from each recruited block one section from those sections measured ten microns was placed in eppendorf tube for PCR method. Each section from the remainder three sections (measuring four microns) was floated in 70% ethanol and water bath (Electrothermal China) at 40c, consecutively. Each floated section was mounted on positive charge immune slide (Thermo Scientific Italy) to detect immune expression of HPV, HSV-2 and EBV in each sample. All slides contained sections were dried in dry oven (WTC binder 7200 TUTTLINGEN, B28, NO.88485-USA) at 60c for 30 minutes.
Methods of detection

Paraffin wax sections were detected using immunohistochemistry and PCR techniques. For IHCAb-3 (Clone K1H8) mouse monoclonal antibody biomarker was used to detect presence of HPV type (6, 11, 16, 18, 31, 33, 42, 51, 52, 56 and 58). LMP-1 biomarker was used to detect expression of EBV. Ab-1 rabbit polyclonal antibody biomarker was used to detect HSV-2. Each antibody used was specific to only one virus and does not cross react with other viruses. All used biomarkers come from Thermo Scientific (Italy). PCR machine (S/N 1320300144, Model LabCycler 48, SensoQuest GmbH D-37085, Hannah-Vogt-Germany) was used to detect HR-HPV, HSV-2 and EBV infections in paraffin sections using specific primer to each virus. Each primer used was specific to only one virus.

Methods of staining

Haematoxylin and Eosin (H&E) staining method

Paraffin section was dewaxed in xylene for 10 minutes, then hydrated through descending grades of alcohol to water, after that stained with Mayer’s hematoxylin for 7 min, washed well in running tap water until section was blued for 5 min, the blued section was counterstained in 1% eosin for 3 min, washed in running tap water for 1 min, dehydrated in ascending grades of alcohol, dehydration was completed by air. The dehydrated section was cleared in xylene; the cleared section was mounted in Disterene, a plasticizer (polystyrene) and xylene (DPX). All mounted sections were examined using Olympus microscope (CX21FS1, Olympus Corporation, Tokyo-Japan). Then the H & E stained sections were reviewed by an experienced histopathologist to include represented samples and exclude the non-represented samples and to ensure that the preceding sections contained representative tissue for IHC and PCR analysis.

Immunohistochemistry (IHC) methods

Sections for IHC technique were stained according to the manufacture instructions (Thermo Scientific- Italy) and then diagnosed by two histopathologists independently.

Paraffin sections from both case and control samples were deparaffinized in two changes of xylene for 10 minutes in each change, then rehydrated in descending changes of ethanol as follows; sections were placed in two changes of absolute ethanol for 5 minutes in each change and then were placed in 90% ethanol for 3 minutes, and then placed in 70% ethanol for 2 minutes, then washed in distilled water for 2 minutes and washed two times in buffer. After rehydration antigens were retrieved in preheated water bath at 95oc in plastic coplinjar contained 1ml Target Retrieval Buffer (TRB) and 50 ml Distilled Water (D.W) of 9 pH for 40 minutes. After antigen retrieval, slides were washed in Phosphate Buffer Saline (PBS) of pH 7.4 for 3-5 minutes, then endogenous peroxidase activity was blocked in hydrogen peroxide block for 10-15 minutes, then slides were washed in PBS for 3-5 minutes and then Ultra V block was applied to each section for 8-60 minutes at room temperature to block nonspecific background staining, then all slides were drained for a few seconds and wiped around the sections with tissue paper and encircled round the tissue using cytomation pen, then specific primary antibody to each virus was applied to each section for 30 minutes, then slides were washed in PBS 3-5 minutes, then the second layer antibody biotinylated goat-anti-mouse/rabbit immunoglobulins was placed on each section for 30 minutes at room temperature, the slides then were washed in PBS for 10 minutes, the third and final antibody layer, streptomyces Avidin Biotin Complex-Horse Radish Peroxidase (StABC/HRP) was placed on each section for 30 minutes at room temperature, the slides then were washed in PBS for 10 minutes. After that 1 drop (40 micro liters) from 3.3 diaminobenzidine tetrahydrochloride (DAB) Plus Chromogen added to 2 ml of DAB plus substrate, mixed by swirling and applied to tissue for 10 minutes, then all slides were rinsed in running tap water (RTW) for 5 minutes, counter stained in Mayer's Haematoxylin for 1 minute, blued in RTW for 5-10 minutes, dehydrated in ascending grades of ethanol, cleared in xylene and mounted in DPX.
Polymerase Chain reaction (PCR) method

Sections for PCR were cut using Leica microtome. All PCR processing methods were done at Institute for Endemic Diseases at Khartoum University.

DNA extraction from histological paraffin sections

Ten microns sections from each tissue block were cut with a microtome and then placed in a labelled Eppendorf tube. An empty paraffin block was sectioned in between tissue blocks to avoid cross-contamination. The DNA was extracted from each tissue sample according to Ron's Tissue DNA Mini Kit (Germany). To each Eppendorf tube contained sections; 250 microlitres buffer PB incl., and 20 microlitres proteinase K were added. The tube then was vortexed in vortex machine (SCIENTIFIC INDUSTRIES, INC. S/N 96406, Model G-560E- BOHEMIA, N. Y., 11716- USA) thoroughly at maximum speed for 15 seconds. The tube was incubated in water bath at 52 oC until tissue completely digested and vortexed occasionally. Some samples showed residual undigested material so they centrifugated in (ZENTRIFUGEN D-78532, Model 2002, Tuttlingen-Germany) at full speed for 2 minutes, and the supernatant was transferred to new Eppendorf tube with the same label. To each supernatant 250 microlitres buffer AB was added and vortexed for 5 seconds. Then solutions were transferred to a spin column by pipetting. Then loaded column centrifuged at 13.000 rpm for 30 seconds. Discarded flow through. Then 400 microlitres buffer WB was added to the spin column. Loaded column Centrifuged at 13.000 rpm for 30 seconds. Discarded flow through. Then the spin column was washed with 400 microlitres of 70% ethanol by centrifugation at 13.000 rpm for 3 minutes. Carefully, the tube was removed and discarded flow-through. Then the column was transferred to new 1.5 ml Eppendorf tube, 75 microliters buffer EB preheated to 70o C was placed in the centre of the column, the lid was closed and incubated for 1 minute. Thereafter, the loaded column was centrifuged at 13.000 rpm for a 1n minute to elute the DNA. Thereafter, the column was discarded. The purity and concentration of the extracted DNA was measured using Nanodrop spectrophotometer, (Model ND 1.000, S/N 02013859SG. USA). The quality of the extracted DNA for PCR was assessed targeting the 585 bp exon 5 fragment of the human Glucose 6 Phosphate Dehydrogenase (G6PD gene) [figure 1] by using the following set of primers:

Forward primer 5'-CTGCAGTTTCTCCGCAATC-3'
Reverse primer 5'-AGGCAACGGCAAGCCTTAC-3'

The obtained DNA was of optimum purity and concentration for PCR. The optimized DNA was stored at -20o C for subsequent PCR analysis.

Viral DNA detection

The presence of HSV, EBV and HPV was detected by PCR. The primer sets used for each virus have been shown to be specific and do not cross amplify other viruses. Detection of HSV, EBV and HR-HPV, was achieved by using a ready mix from Sacace biotechnologies (Italy).

HSV-2 PCR detection

HSV-2glycoprotein (the target DNA) was detected by PCR using specific primers mixed with ready mix from Sacace biotechnologies, the primers sequences were not provided.

(Amplicon (bp) was 430) [figure 2].

Ten L of PCR-mix-2 were added to each PCR-mix-1 tube to the surface of wax of each tube ensuring that it was not fall under the wax and mixed with PCR-mix-1. Then to appropriate tube to the surface of wax 10 L of optimized DNAwere added.PCR-mix-1 tubes were closed and transferred into the thermalcycler only when temperature reached 95°F and the following program was followed:

For HSV-2 an initial denaturation step was 95°C for five minutes followed by 42 cycles of 10 seconds each for secondary denaturation (95°C), annealing (65°C), and extension (72°C), with one
minute additional extension step at 72°C after the last cycle.

**EBV PCR detection**

An EBV latent transmembrane protein (LMP-2) was detected by PCR using the following primers:

Forward primer:5’-GTGTGCGTGTCGCGGCGCCAGCCAC-3’
Reverse primer:5’-ACCTGGGAGGGCCATCGCAAGCTCC-3’

(Amplicon (bp) was 375) [figure 3].

Briefly, 5 μl of the extracted DNA were amplified in already Maxime PCR PreMix (iTaq, for 20 μl 1 reaction mixture containing iTaq TM DNA Polymerase (2.5U), dNTPs 2.5mM, PCR buffer (1x) and gel loading buffer (1x) (iNtRON Biotechnology, Senognam, Korea) and 1 μl forward, 1 reverse from each primer, 18 μl D.W.

For EBV an initial denaturation at 95°C for three minutes (1 cycle) followed by 10 cycles of 30 seconds each for secondary denaturation (94°C), first annealing at 70°C (10 cycles) 45 seconds for each cycle, and extension (72°C) for 2 minutes, followed by second annealing at 63°C (15 cycles) 45 seconds for each cycle, with ten minutes additional extension step at 72°C after the last cycle.

**HR-HPV PCR detection**

E1, E2 genes conservative regions of HR-HPV type (16, 18, 31, 33, 35, 39, 45, 52, 53, 56, 58, 59, 66, 70), were detected by PCR using specific primers mixed with ready mix from Sacace biotechnologies. For each sample in the new sterile tube 5 μl of PCR-mix-1, 10 μl of 2, 5x buffer and 0, 5 μl of Taq Polymerase were added to prepare Reaction Mix. Then 15 μl of Reaction Mix was added to each sample tube. Thereafter 1 drop (25 μl of Mineral Oil) was added to each sample tube. Then to appropriate tube 10 μl of qualified DNA sample was added. (Amplicon (bp) was 267-325 bp) [figure 4].

For HPV an initial denaturation at an initial denaturation step was 95°C for fifteen minutes followed by 42 cycles of 30 seconds for secondary denaturation (95°C), 40 seconds for annealing (63°C), 40 seconds for first extension (72°C), with one-minute additional extension step at 72°C after the last cycle.

To avoid possible cross-contamination, all PCR reactions were carried out under stringent conditions. A negative control was included after each fifth sample.

**Gel electrophoresis**

Five μl of the PCR product was placed in well of gel electrophoresis (S/N 0706.11016 Biometra GmbH, Rudolf-Wissell-Straße 30, D-37079 Göttingen-Germany) for 1 hour (V 96, A 34). After that gel electrophoresis was estimated under ultraviolet using Gel documentation (S/N BUV025080832/1504-586, Biometra GmbH, Rudolf Wissell-Straße 30, D-37079 Göttingen-Germany) to detect presence or absence of virus.

**Quality control:**

Quality control measures have been taken in all steps to prevent carry-over contamination by using microtome knife for each case, cleaning thoroughly the microtome and the water path and examining the slides. Manufacturer instructions were followed strictly. Control samples were used.

**Statistics:**

All obtained results were analyzed by Statistical Package for the Social Sciences (SPSS) version 22.0, with Pearson’s chi-square test used to assess intergroup significance. Other variables, frequencies, odd ratio and CIs were calculated for comparison and presented in form of figures and tables. P value and odd ratio were used to assess the significance of the results.
Results

A total of 98 samples from cases patients with histopathologically confirmed cervical squamous cell carcinoma were taken as cases and 82 cervical samples from patients having problems other than carcinoma were taken as controls. The age of patients was ranged from 24-80 years with average mean of 55 years. Using both techniques PCR and IHC, HPV was found to be positive in 40/98 (41.8%) among case group while in the controls reported in 2/82 (2.4%) of the samples. There was a high statistically significant difference. The P value was 0.000. The odd ratio was 28.772, CI=6.686-123.86. The PCR results of HPV infection among case group out of the 98 cases, HR-HPV was identified in 41/98 (41.8%), of the cervical tissues. The IHC results of HPV infection revealed out of the 98 cases, HPV was identified in 28/98 (28.6%), of the cervical tissues. The situation of HSV-2 was like that it was positive in 6/98 (6.1%), while the healthy individuals reported in 1/82 (1.2%) positive rate, with no statistic significant difference. The P value was 0.128. PCR revealed HSV-2 infection among the case group, HSV-2 was identified in 2/98 (2%), of the cervical tissues. whereas IHC revealed infection in case group 6/98 (6.1%), of the cervical cancer tissues.

The correlation of HSV-2 coinfection with HPV in cervical SCC. Of the 41 HPV infected samples HSV-2 was detected in 4/41(10%) among cases, coinfection was not detected in control samples.

The situation of EPV among cases was like that, it was positive in 2/98 (2%), while the healthy individuals reported in 0/82 (0.00%) positive rate, with no statistic significant difference. The P value was 0.501. The correlation of EPV coinfection with HPV in cervical SCC. Of the 41 HPV infected samples EPV was detected in 1/41(2.4%) among cases, coinfection was not detected in control samples.

Table 1: HPV testing results among case & control groups.

<table>
<thead>
<tr>
<th>Methods of detection</th>
<th>Case group</th>
<th>Control group</th>
<th>Total</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>PCR</td>
<td>40</td>
<td>58</td>
<td>Zero</td>
<td>82</td>
</tr>
<tr>
<td>IHC</td>
<td>28</td>
<td>70</td>
<td>2</td>
<td>80</td>
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</table>

Chi square test  
Power of significant is 0.05

Table 2: HSV-2 testing results among case & control groups.

<table>
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<th>Methods of detection</th>
<th>Case group</th>
<th>Control group</th>
<th>Total</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>PCR</td>
<td>2</td>
<td>96</td>
<td>Zero</td>
<td>82</td>
</tr>
<tr>
<td>IHC</td>
<td>6</td>
<td>92</td>
<td>1</td>
<td>81</td>
</tr>
</tbody>
</table>

Chi square test  
Power of significant is 0.05
### Table 3: EPV testing results among case & control groups.

<table>
<thead>
<tr>
<th>Methods of detection</th>
<th>Case group</th>
<th>Control group</th>
<th>Total</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>PCR</td>
<td>Zero</td>
<td>98</td>
<td>Zero</td>
<td>82</td>
</tr>
<tr>
<td>IHC</td>
<td>2</td>
<td>96</td>
<td>Zero</td>
<td>82</td>
</tr>
</tbody>
</table>

*Chi square test*
*Power of significant is 0.05*

### Table 4: Frequency of HSV-2 coinfection with HPV:

<table>
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<th>Virus coinfection</th>
<th>Study group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Case</td>
<td>Control</td>
</tr>
<tr>
<td>HSV-2+HPV</td>
<td>Positive</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>37</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>41</td>
</tr>
</tbody>
</table>

### Table 5: Frequency of EBV coinfection with HPV:

<table>
<thead>
<tr>
<th>Virus coinfection</th>
<th>Study group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Case</td>
<td>Control</td>
</tr>
<tr>
<td>EBV+HPV</td>
<td>Positive</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>40</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>41</td>
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</tbody>
</table>

### Discussion

This is the first study to explore the co infection of HPV, HSV-2 and EPV in cervical squamous cell carcinoma in Sudan. Actually the role of HPV was investigated and documented in different studies in the country. High risk HPV were proved to have part in the causation of cervical lesions within the range of LCIN, HCIN, CIS and ISC in addition to this new types were recognized like type 31 (unpublished data). This study further adds prove to the role of HPV and to the sensitivity of PCR technique in detection of HPV HSV-2 and EPV infections. Concerning HPV infection it was found to be positive in 40.8% and 28.6% of cervical cancer cases by PCR and IHC respectively. These values are much lower compared to that recorded by Sigrun et al., 2007, Abdelhalim et al., 2013 and Pavi et al., 2006. However our study was in agreement with the study conducted in our country by Abdelbaset et al., 2012. Relative similar results were obtained in Polish study by Dybikowska et al., 2002. This study showed much lower incidence of HPV among cervical cancer in Sudanese women than among other populations. In a country like Sudan where cervical cancer pose a big burden and usually present in late stage, it is an unexpected finding. At the same time it gives way to look for other causes. This may be due to variation in geographic regions and population groups. Also the lower ratio of HPV infection among Sudanese women with SCC may indicate other causes of cervical cancer like long term use of oral contraceptives, intrauterine device use, multiple full-term pregnancies, young age at full-term pregnancy, poverty, socioeconomic status, having a weakened immune system,
chronic inflammation, hormonal factors, immunosuppression, diet, diethylstilbestrol and family history of cervical cancer.

Our results revealed that; there was no statistically significant correlation between the two viruses and development of cervical cancer. Regarding HSV 2 coinfection with HPV; we determined that; in case samples the coinfection was higher than in control samples (frequency 4/41[10%], for cervical squamous cell carcinoma). The obtained results indicated that the presence of HPV is associated closely with cervical cancer, and that HSV 2 infection or co-infection with HPV might be involved in cervical cancer development. Similar result obtained by (You et al., 2012) they concluded that HSV 2 coinfection with HPV in cervical squamous cell carcinoma was strongly higher than in healthy women (ORs = 61.1, P < 0.01 for squamous cell carcinoma). Our result nearly similar to that study conducted in Poland by Kwansniewska et al., 2009, they showed that; the prevalence of co-infection of HPV with HSV-2 in cervical cancer patients was more than control individuals. Nearly similar result obtained by (Szostek et al., 2009) they concluded that; in the 60 cervical HPV-16-positive samples studied, among them HSV-2 was detected in 4%. Our result different to that obtained by (Di Luca et al., 1987; Di Luca et al., 1989) they summarized that; six of eight cervical cancer biopsy specimens that contained HSV-2 DNA sequences also contained HPV DNA. Different result was obtained by (Pérez et al., 2006) they concluded that; herpes simplex prevalence in HPV positive (20.8%) women was approximately the same as in negative (21.8%) women. Different result was obtained by (Paba et al., 2008) they concluded that; of the 136 HPV DNA positive cervical cancer tissues; 32/136 contained also HSV2 DNA.

Regarding EBV coinfection with HPV we found that; in case samples the coinfection was present in only one sample, while the control samples were found free of coinfection. The obtained results indicated that the presence of HPV is associated closely with cervical cancer, and that EBV infection or co-infection with HPV might not be involved in cervical cancer development. Similar results were obtained by other authors (Hf-Rding et al., 1992; Hilton et al., 1993; de Oliveira et al., 1999; Lanham et al., 2001; Yang et al., 2004) they concluded that; there was no EBV infection in cervical cancer specimens as well as HPV positive samples. Relatively different result obtained by (Szostek et al., 2009) they concluded; in the 60 cervical HPV-16-positive samples studied, among them EBV was detected in 22% of the samples. Our result also different to that obtained by (Abdelhalim et al., 2013) they concluded that; the highest co-infection (HR-HPV and EBV) was found in squamous cell carcinoma cases (67%).

Conclusion

The frequency of infection with HR-HPV subtypes is high among Sudanese women with cervical SCC and suggests a role of HR-HPV in the development of cervical cancer in Sudan.

HSV-2 is detected in cervix tissue with SCC but in small number and this suggest its role as bystander rather than risk factor for developing cervical cancer.

There is no statistical significant correlation between EBV and tumorigenesis of cervical SCC

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