

SCREENING FOR HIGH RISK HUMAN PAPILLOMA VIRUS (HR-HPV), CYTOMEGALOVIRUS, HERPES SIMPLEX VIRUS (HS-2), AND EPSTEIN - BARR VIRUS AMONG SUDANESE FEMALES WITH CERVICAL CANCER

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BACKGROUND: Cervical cancer is one of the commonest female cancers in Sudan due to the absence of HR-HPV screening and vaccination programs. The aim of this study was to screen for the existence of cervical cancer related herpes viruses (HPV, EBV, CMV and EBV) among Sudanese women with cervical cancer.

METHODOLOGY: This was analytical 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. Of the 180 tissue blocks, 98 blocks were from patients with cervical squamous cell carcinoma (case group) and 82 were from cervical tissue blocks malignancy-free (control group). Screening for herpes viruses (HPV, EBV, CMV and EBV) were performed using Immunohistochemistry (IHC) techniques.

RESULTS: HR-HPV infection was detected in 28.6% of the cases, and 2.4% of the controls. Association between HR-HPV and cervical carcinoma was found to be statistically significant $P < 0.0001$. CMV infection was detected in 15.3% of the cases and 1.2% of the controls with statistically significant difference, the $P < 0.0001$. HSV-2 revealed positivity in 6.1% of the cases and in 1.2% of the controls with no statistically significant correlation. EBV was positive in 2% of the cases, while none of the control was found positive.

CONCLUSION: HR-HPV and EBV are prevalent etiological factors that contribute to the etiology of cervical carcinoma in Sudan. HSV-2 and EBV were insignificantly found in cervical cancerous lesions.

Keywords: HPV, CMV, HSV, EBV, Cervical SCC, IHC, Sudan.

INTRODUCTION:

Cervical cancer is a malignant tumor developing from the ectocervix [1]. It is a result of the abnormal growth of cells that have the ability to invade or metastasis to other sites of the human [2]. Cervical cancer typically arises from dysplastic changes over 10 to 20 years. Worldwide, cervical cancer is both the fourth-most common cause of cancer and the fourth-most common cause of cancer death in females [3]. It is a relatively rare cancer in nations that have instituted and maintained national screening programs. Unfortunately, the screening program has failed in most countries where more than 80% of new cases of cervical cancer are diagnosed. [4]. The causative association between Human Papilloma Virus (HPV) and Squamous Cell Carcinoma (SCC) was established [5].

HPV is not a sufficient factor for cervical cancer tumorigenesis [6], [7]. It is still doubtful what the existence of CMV, HSV-2 and EBV in cervix and if these viruses can act as co-factors in cervical tumorigenesis.

This was the first study in Sudan investigated the role of viruses other than HPV, namely CMV, HSV-2, EBV in the pathogenesis of cervical carcinoma.

MATERIALS AND METHODS:

This was analytical case control study carried out during the period from October 2013 to June 2016. Samples involved in this study were 98 paraffin tissue blocks with cervical squamous cell carcinoma versus 82 blocks without cervical cancer (females underwent hysterectomy due to cases other than cervical carcinoma). The collected tissue blocks obtained from different histopathology laboratories in Khartoum state-Sudan (Maternity Hospital, Military Hospital, Total Lab care, Alrahma Medical Centre and Ribat University Hospital). Convenient sampling technique was used to collect samples.

Data collection tools and variables: Master sheets were used to record all patients and sample data; age, presenting symptom, type of SCC and residence. Master sheets were also used to record IHC results.

Sample processing: One section from each block measures four micrometers was cut using Leica microtome (Leica Microsystems, Nussloch GmbH, model: RM 2125RT, ser NO. 8843/04-2005-China) and then stained in H&E to confirm diagnosis of each block.

Then four sections were cut from each recruited block using the same microtome, Each section from the four sections (measuring four microns) was floated in 70% ethanol and water bath (Electrothermal ser NO.18861434-China) at 40°C, consecutively. Each floated section was mounted on positive charge immune slide (Thermo Scientific- Italy) to detect immune expression of HPV, CMV, 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 60°C for 30 minutes.

Methods of detection: Paraffin wax sections were detected using immunohistochemistry techniques. For IHC Ab-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 antigens while CMV was detected using specific a mixture of 2 mouse monoclonal antibodies that react with immediate early and early protein antigens in tissues infected with CMV. 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).

Methods of staining:

Haematoxylin and Eosin (H&E): The H&E stained sections were subjective to include represented samples and exclude the non-represented samples. The included H&E sections were used to ensure that the preceding sections contained representative tissue for IHC analysis. H & E staining method was achieved according to standard procedure of Mayer's Haematoxylin technique.

Immunohistochemistry (IHC) methods: Sections for IHC technique were stained and diagnosed in histopathology lab at Sharq Elneil College and reviewed at National Ribat University 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 95°C 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 cytostation pen, then specific primary anti-

body 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.

RESULTS:

A total of 98 cases (patients with histopathologically confirmed cervical squamous cell carcinoma) and 82 controls (patients apparently normal) were included in this study. The age of patients was ranged from 24-80 years with average mean of 55 years. The age of control groups was ranged from 23-80 years with average mean of 51 years. The age distribution was relatively similar between cases and controls. Most of cervical cancer patients were in the sixth decade (29.6%), followed by fifth and seventh decades (25.5%) as indicated in Figure 1.

Figure 2 summarizes the frequency of presenting symptoms among patients with cervical cancer, of the 98 cases, vaginal bleeding was identified in 73/98 (74.5%), followed by cervical mass and vaginal discharge constituting 19/98 (19.4%), 3/98 (3.1%) respectively.

Table 1. Summarizes the IHC results of HPV infection. Of the 98 cases, HPV were identified in 28/98 (28.6%), of the cervical tissues, and the CMV infection of the 98 cases, CMV was identified in 15/98 (15.3%), of the cervical tissues also in the HSV-2 infection of the 98 cases, HSV-2 was identified in 6/98 (6.1%), of the cervical cancer tissues. and the results of EBV infection. Of the 98 cases, EBV was identified in 2/98 (2%), of the cervical cancer tissues.

DISCUSSION:

Persistent infection by oncogenic types of HPV is considered as etiological factor for cervical carcinoma development. Although HPV is not a sufficient factor for developing the cancer, several other co factors were identified, such as: infection by other sexually transmitted diseases (STI) (HIV, Chlamydia trachomatis, Cytomegalovirus, [6], [7] and Herpes Simplex Virus (HSV-2 [8]. There is an increasing demand to characterize development of cervical cancer and whether the co-infection of CMV, HSV-2 and EBV play a role in the pathogenesis of cervical cancer. →

Figure 1: The description studied subjects by age

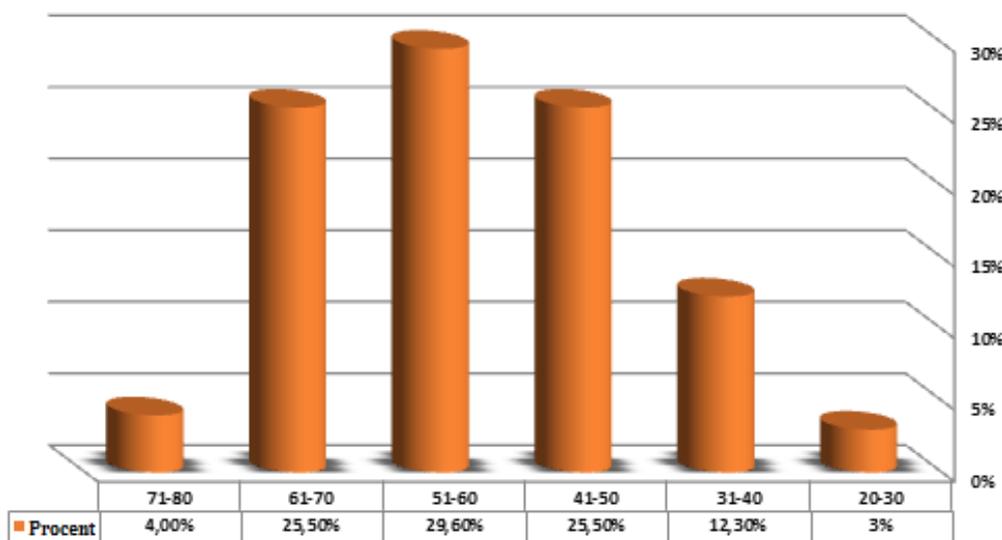


Figure 2: The Frequency of presenting symptoms in the studied patients

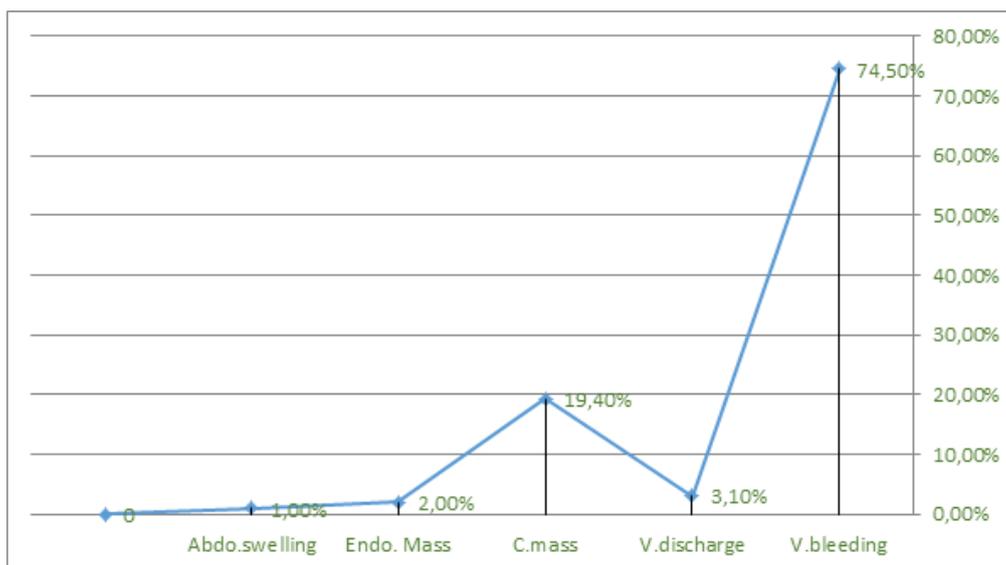


Table 1: Frequency of HPV, CMV, HSV-2 and EPV infection among case group by using IHC

	Frequency		%	
	Lower	Upper	Lower	Upper
HPV	28	70	28,6	71,4
CMV	15	83	15,3	84,7
HSV-2	6	92	6,1	93,9
EPV	2	96	2	98

The Present study aimed to investigate the CMV, HSV-2 and EBV as co-infection with HR-HPVs by using IHC methods among cervical squamous cell carcinoma, as the first time in Sudan. The current study showed that most of cervical cancer patients were in the higher age; this is in keeping with the natural history of HPV infection.

The mean age at presentation was 55 years, which were higher compared to studies conducted by Badar et al., 2007 [9] and Reimers et al., 2009 [10], but similar to studies done by Krishnamurthy et al., 1997 [11], Herbert et al., 2001 [12] and Patel et al., 2009 [13]. Concerning HPV infection among cervical cancer, the positive rate was 40.8% and 28.6% detected by IHC respectively, while only two samples from control group showed immunostain positive reaction with statistically significant difference. Our results established strong correlation between HPV and cervical cancer as the P value was 0.000 and the odd ratio was 28.772. Our results are lower in comparison to that obtained by Sigrun et al., 2007 [14] they concluded that IHC analysis of biopsies from 58 patients with invasive cervical SCC revealed fifty-five (95%) of the 58 SCCs were HPV-DNA-positive. Also lower to that obtained by Abdelhalim et al., 2013 [15] they concluded that HR-HPV infection was found in (88%) in patients with SCC, and lower to that obtained by Pavai et al, 2006 [16]. This study showed much lower incidence of HPV among cervical cancer in Sudanese women than among other populations. This may be due to variation in geographic regions and population groups [17]; [18].

analyte biological action of HPV in development of cervical cancer, and concluded that; according to the current model, the initial events of cervical carcinogenesis after viral infection are that high-risk HPV types undergo specific changes which overcome the transcriptional control of viral gene expression in the infected

keratinocytes. Inactivation of these cellular control functions permits deregulated transcription of the early viral genes E6 and E7, and that triggers cell proliferation, inhibition of apoptosis, reprogramming of differentiation, and chromosomal instability. These changes could support the integration of episomal HPV genomes into

chromosomes of the host cell, and contribute to further over expression of the viral genes E6 and E7, resulting in an increase of the E7 oncoprotein levels during early steps of cervical carcinogenesis. The high-risk E7 protein, in cooperation with high-risk E6, can efficiently immortalize human primary keratinocytes; and the consistent over expression of the E6 and E7 oncogenes is required to induce and maintain the transformed phenotype of cervical cancer cells. Immortalization by the E7 oncoprotein involves its ability to bind and thereby functionally inactivate cell cycle-regulatory proteins like the retinoblastoma tumor suppressor protein. Further work has shown that E7 acts as a multifunctional protein, deregulating several additional cellular pathways necessary for the oncogenic potential of the virus [19]; [20]; [21].

Cytomegalovirus is recognized as one of the most frequent viruses to infect the genital tract. Nevertheless, there is no study that clearly characterizes CMV infection in cervical epithelium.

The present study showed overall frequency of CMV infection positive rate was 23.5 % in cervical cancer, considering HPV coinfection; we observed CMV infection in 22 % among HPV positive women. These results indicate that; CMV may act as a cofactor with HPV to develop cervical cancer and also may be a risk factor for cervical cancer. Our result similar to that result in china included in systemic review study conducted that; CMV was detected in 12 cases (21.8%) out of 55 patients with CIS & ICC , also similar to that study conducted in Portuguese Institute of Oncology of Porto by Marinho et al., 2013 [22]; they concluded that; CMV was detected in 22.2% of in situ/invasive carcinomas. Our result was relatively near to that study conducted in Uganda by Odida and Schmauz, 1996 [23]; they concluded that; CMV was detected in 5 out of 34 cases of cervical cancer using immunohistochemistry. They summarized that; In CIS/ICC cases, the analysis revealed a CMV frequency of 48.6% in Europe and 41.2% for Asia, with a global rate of 44.4%. In Poland CMV was detected in 18 cases (78.3%) out of 23 patients with CIS & ICC. In Greece CMV was detected in 17 cases (34.7%) out of 49 patients with CIS & ICC. In south-East Asia CMV was detected in 40 cases (41.2%) out of 97 patients with CIS & ICC. In Thailand CMV was detected in 28 cases (66.7%) out of 42 patients with CIS & ICC. Regarding HSV 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 [24], 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 [25], 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 [26], 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 [27]; Di Luca et al., 1989 [8], 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 [28], 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 [29]; they concluded that; of the 136 HPV DNA positive cervical cancer tissues; 32/136 contained also HSV2 DNA. Regarding EBV result was concur to that obtained by Yang et al., 2004 [30], they summarized that; among 27 biopsies of cervical cancer, EBV was not found in the cervical cancer samples and might have no or little relationship with cervical cancer. Also similar to that obtained by Lanham et al., 2001 [31], they concluded that; EBV DNA was only detected in two (2.3%) samples with cervical cancer. Similar result was also obtained by de Oliveira et al., 1999 [32]. they concluded that; of 65 cervical sample among which 5 with CIN-III, 30 with Invasive carcinoma of cervix, 3 with Well differentiated SCC, 7 with Moderately differentiated SCC, 14 with Less differentiated SCC, 6 with Lymphoepithelioma-like carcinoma., there was a lack of EBV infection in cervical carcinomas. Also similar result obtained by Hf-Rding et al., 1992. They concluded that; Epstein-Barr virus DNA was demonstrated in none of the 22 cervical carcinomas. Also similar to that study obtained by Hilton et al., 1993 [33]. they observed absence of EBV in carcinoma of the cervix. Relatively similar result obtained by Thaís et al., 2015 [34] they concluded that; HPV frequency was associated with cervical cancer cases ($p = 0.005$) but not EBV frequency ($p = 0.732$). These observations suggest a positive association between EBV and carcinoma of the cervix (Se et al., 1993) [35].

CONCLUSION:

On the base of the obtained results we could conclude that;

- The mean age of patients presenting with cervical cancer is 55 years. This is in keeping with the natural history of the HPV.
- Vaginal bleeding is the presenting symptom observed in more than two thirds of patients with cervical SCC.
- Large non-keratinizing SCC is predominant type over the keratinizing SCC.
- The overall viral infection is observed in 60% of SCC samples versus 5% in cancer-free cervix tissue. This suggests that viral infection play a role for developing this type of cancer.
- 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.

- The high rate of HR-HPV and CMV co-infection in SCC suggests that CMV infection is incriminated in cervical cancer progression. This could be taken into account as bad prognosis in this type of cancer.
- 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.
- CMV coinfection with HPV infection and HSV-2 coinfection with HPV is high among cases in comparison to control group.

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