Low CD4 cells and viral co-infection increase the risk of VaIN: Use of SCCA1 and Ki67 as diagno-prognostic biomarkers

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A B S T R A C T

This study evaluated the correlation of SCCA1, Ki67 and CD4 cell expressions and classified vaginal smears in individuals co-infected with Human immunodeficiency virus (HIV), Herpes simplex virus 2 (HSV2), Epstein Barr virus (EBV) and Human Papilloma virus (HPV). This cross sectional study included 173 participants within the age range of 20–70 years. Vaginal smears were stained by Papanicolaou technique and classified into high-grade squamous cell intraepithelial lesion (HSIL), low-grade squamous intraepithelial lesion (LSIL), atypical squamous cells of undetermined significance (ASCUS) and negative for intraepithelial lesion (NIL). Presence of immunoglobulin M and G antibodies for EBV, HIV, HPV and HSV2, and SCCA1 and Ki67 antigens were determined by ELISA method. Result showed that biomarkers SCCA1 had higher sensitivity (87.5%) to vaginal lesions when compared with Ki67 which had a sensitivity of 70.8% (p > .01). Assays revealed viral co-infections of 96.0% and 16.8% in smears positive and negative for vaginal lesions, respectively (p < .01) with HIV, HSV2 and EBV as the most prevalent type of co-infection (36%). The findings of this study suggest that low CD4 cells and viral co-infection could increase the risk of developing vaginal lesions. This study also suggests that SCCA1 and Ki67 could be used as diagnostic and prognostic biomarkers for vaginal intraepithelial neoplasia (VaIN).

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1. Introduction

Vaginal cancer is a malignant growth on the epithelial lining of the vagina and comprises of approximately 1–3% of all gynecologic malignancies worldwide [10,11–14]. Cancer of the vagina which can either be primary or metastatic are linked to risk factors including: smoking of cigarette, human immunodeficiency virus (HIV), herpes simplex virus 2 (HSV-2), Epstein Barr virus (EBV) and Human Papilloma virus (HPV) infections [3] and history of other malignant urogenital diseases. Herpes simplex virus 2 has been reported to increase HIV acquisition in men and women [4]. Human immunodeficiency virus (HIV) infected individuals are at a greater risk of some variants of cancer compared with uninfected individuals who are within the same age range [3]. This is because infection with HIV adversely affects the body's immune system and diminishes its ability to eliminate infecting organisms, especially viruses which can increase the risk of cancer [6,7]. Report has it that HPV types increase the chances of HIV infection in Africa [8]. The high prevalence of HIV in Ogun State (11.7%) as reported by Motayo et al. [9] as against the national value of 3.34% [10] suggests that vaginal cancer could be high in the State. Furthermore, HSV-EBV co-infection has been implicated in anal cancer especially among individuals co-infected with HPV + HIV [11–14]. However, to this day, there is little or no information on the prevalence of such viral co-infections in vaginal lesions, particularly in Nigeria. This could be due to poor surveillance and monitoring of associated clinical presentations in the country [15]. Thus, this study is warranted.

Proliferation antigen, Ki67, found in the nuclei of developing cells, has been used as a marker of cell proliferation and for grading dysplastic anal, cervical, vaginal and vulva biopsies [16,17]. On the other hand, squamous cellular carcinoma antigen (SCCA), an aggressive cancer marker, is a member of the (serpins) serine protease inhibitors. High serum and tissue levels in hepatocellular carcinoma, head and neck, cervix, lung, oropharynx and other epithelial cancers have been reported [18]. Still, no study has attempted to use and compared the diagnostic value of SCCA1 and Ki67 in grading vaginal smears, hence the need for this study.

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2. Material and methods

2.1. Study area, inclusion and exclusion criteria

This study was carried out at the HIV clinic in State Hospital Ija, Abeokuta (latitude 07° 03’N and longitude 03° 19’E) which serve people in Ogun state and other bordering states with a population of 494,700 in the year 2015 [19]. In this cross-sectional study, a total of 173 female participants (103 HIV seropositive and 70 HIV seronegative participants) within the age range of 20–70 years were included using the non-probability sampling technique, irrespective of any clinical features of malignancy. However, participants who were diabetic, pregnant, were menstruating, had undergone radiation therapy or chemotherapy, had hysterectomy, organ transplantation or have been diagnosed of any other cancer other than gynaecological cancers were excluded from this study. The HIV infected participants who were recruited in this study were all on highly active anti-retroviral therapy (HAART).

2.2. Blood sample collection and assays

Six (6) ml of whole-blood samples were collected from participants by venipuncture. Three ml were discharged into an EDTA vacutainer while three ml were discharged into a plain tube and allowed to stand for two hours (for clotting), sera were separated into plain bottles and stored at –20°C until when analyzed. The EDTA whole-blood samples were investigated for TCD4 cells using CD4 easy count kit (Sysmex Partec GMBH, Germany) and Sysmex Partec Cyflow Counter IVD flow cytometer. Separated sera were tested, using commercial ELISA kits, for IgG and IgM antibodies of EBV (Calbiotech Inc, El Cajon, USA), HIV (Qingdao Hightop Biotech Co, Ltd, China), HSVII (Qingdao Hightop Biotech Co, Ltd, China). Antibodies for HIV-1 and HIV-2 and P24 antigen were detected using ELISA kit (Qingdao Hightop Biotech Co. Ltd, China). Quantitative determination of proliferation-related Ki-67 antigen concentration was carried out by ELISA kit (Melsin Medical Co, Ltd, China) while that of Human Squamous Cell Carcinoma Antigen 1 (SCCA1) was also carried out by ELISA kit produced by Elabscience Biotechnology Inc, USA. All investigations were carried out according to manufacturers’ instruction. All laboratory investigations were carried out at the laboratory complex of Babcock University Teaching Hospital.

2.3. Vaginal sample collection, handling and classification of smears

Samples were collected from the vagina using Ayres spatula following dilation of the external genitalia with disposable speculum and Colposcopy. Smears were made from the Ayres spatula on labelled, grease free, clean slides, spray-fixed accordingly and stained by the Papanicolaou techniques. The stained vaginal smears were classified by two pathologists based on the recommendations of the International Society for the Study of Vulvovaginal Disease Terminology: 1. Negative for intraepithelial lesion or malignancy (NIL), 2. Atypical squamous cells of unknown significance (ASCUS), 3. Low-grade squamous intraepithelial lesion (LSIL), 4 High-grade squamous cell intraepithelial lesion (HSIL). Participants with positive smears (HSIL) were biopsied for confirmation.

2.4. Statistical analysis

Descriptive statistics were carried out on the data and are given as frequency distribution for categorical variables. Data generated were analyzed by Chi-square or Analysis of variance using statistical package for social sciences (SPSS) version 16. Significance levels were set at p < .01 and p < .05.

2.5. Ethical consideration

Ethical approvals were obtained from Babcock University Health Research and Ethics Committee (BUHREC665/16) and State Hospital Abeokuta Ethics Committee (SHA/RES/VOL2/147) before the commencement of this research work. Written informed consent was also obtained from the participants before the collection of samples and other relevant information. Participants who were positive for vaginal lesions were informed, counseled and referred to gynecologist for expert management.

3. Results

Table 1 below shows that the prevalence of LSIL and ASCUS are higher among HIV seronegative participants compared with HIV seropositive participants (p > .05). This suggests that HAART may have conferred immunity against some oncogenic virus. However, the table reveals that the prevalence of HSIL is more frequent among HIV positive when compared with HIV seronegative participants (p > .01). This suggests that other factors, possibly genetic factors, other than HIV may have favoured the development of HSIL. Overall, the prevalence is vaginal lesions are higher among HIV seropositive participants as shown by their lower percentage of negative vaginal smears (85.4%) when compared with that of their seronegative counterparts (p > .05). Biomarkers SCCA1 and Ki67 had sensitivities of 87.5% and 70.8%, respectively. This suggests that SCCA1 is a better biomarker than Ki67 in detecting vaginal lesions. Despite the fact that there are some biomarker negatives among the smear positives, both SCCA1 and Ki67 complemented each other in detecting positive smears. Furthermore, SCCA1 and Ki67 are more sensitive to positive vaginal smears of HIV seronegative participants when compared with HIV seropositive participants (p > .05).

Table 2 shows that the average age for the development of HSIL is higher when compared with the age of those with and without other types of vaginal lesions (p > .05). It also shows that those who are positive for HSIL had lower CD4 counts and had their first sexual intercourse at early ages when compared with other groups (p > .05). The insignificant in TCD4 cell counts could be due the fact that all participants who were HIV positive were on HAART. SCCA1 and Ki67 exhibited descending pattern of expression in LSIL, HSIL and ASCUS. The values ≤ 1650 and ≤ 7.849 were used are cut-off values for SCCA1 and Ki67 positivity, respectively. Significant correlation between Ki67 and Age was also observed (p = .023, r = 0.470).

As shown in Table 3, the seroprevalence of IgG antibodies for EBV, HPV and HSV2 were observed highest in HSIL, ASCUS, LSIL while that of IgM antibodies for EBV, HPV and HSV were highest...
in ASCUS (p > 0.05). This suggests that viral infections are associated with premalignant lesions. However, there is a decreasing order in the prevalence of viral cytopathic effect (perinuclear halo) in HSIL, LSIL, ASCUS and NIL. The latter, correlates well with HSV2 IgG antibodies (p = 0.014, r = 0.504). The table also shows that the HIV seropositive participants with vaginal lesions had more IgG for HSV2, EBV and HPV, and IgM antibodies for EBV and HPV but less IgM antibodies for HSV2 when compared with HIV seronegative participants with vaginal lesions (p > 0.01). There is a positive correlation between the levels of CD4 count, SCCA1 and Ki67 (p > 0.05).

Table 4 above shows a decreasing frequency of HIV + HSV2 + EBV, EBV + HIV + HSV2, EBV + HSV2, HPV + HSV2, HSV + HSV2 and HIV + HSV2 coinfections in vaginal lesions. It also shows that ASCUS had higher number of EBV, HIV, HPV and HSV2 coinfections when compared with HSIL. More so, HSIL had higher frequency of HIV, HSV2 and HSV2 coinfections when compared with LSIL and ASCUS. In the entire studied population, there was a decreasing frequency of HIV + HSV2 + EBV, EBV + HSV2, EBV + HIV + HSV2, HIV + HSV2, HPV + HSV2, HSV + HSV2 and HIV + HSV2 coinfections. There were no HIV + HPV, HIV + EBV and EBV + HPV coinfections among the entire participants. This suggests that HSV promotes HIV, EBV and HPV infections. The seroprevalence of IgG and IgM antibodies revealed viral co-infections of 96.0% and 16.8% in smears positive and negative for vaginal lesions, respectively (p < 0.01). Since 96% of participants with viral coinfections had vaginal lesions (as shown in the table above, 16.8% of participants with negative vaginal smears could be positive for vaginal lesions in the near future. The table also shows that HIV seropositive participants had more HSV2 and EBV co-infections (10.4%) when compared with HIV seronegative participants (5.8%).

4. Discussion

This study investigated the expression levels of SCCA1, Ki67 and CD4 cells in relation to viral co-infection and vaginal smear pattern (Figs 1–4). The prevalence of vaginal lesions (VaIN) in HIV seropositive (who were on HAART) and HIV seronegative participants were also investigated.

Our findings revealed slightly higher prevalence of vaginal cancer, 5.8% for HIV seropositive and 1.4% for seronegative participants (Table 1), than earlier reported by Creasman [1] and Okolo et al. [2]. Surprisingly, the average age of participants with HSIL were higher than that of those who had LSIL and ASCUS but lower than that of those who were negative for vaginal lesions (Table 2). This suggests that age might be a risk factor for the development of intraepithelial lesions and its severity. Decreased CD4 count and early age of sexual intercourse have been linked to severe high-grade squamous intraepithelial (HSIL), particularly in HIV seropositive patients and those co-infected with HPV [14,20]. This is in line with our findings as those who were positive for HSIL had the lowest CD4 cells and had early sexual intercourse. The early age of sexual intercourse may have led to HIV acquisition (with concomitant decrease in CD4 cells) which in turn led to co-infection with oncogenic viruses, hence the manifestation of HSILs. Pokomandy et al. [21] reported that HAART has protective effect towards the development of severe intraepithelial neoplasms when administered for more than four years. However, Crum–Cianflone [22] observed that HAART may be less effective in preventing moderate or severe intraepithelial lesion due to high degree of HIV-related immune depletion following other viral coinfections. In such situations, infected patients may have accumulated sufficient genetic alterations which may ameliorate the positive effect of HAART. The latter report might be linked to the high frequency of HSIL observed among HIV seropositive participants (despite the fact that they have been placed on HAART) when compared with the HIV seronegative participants (who were not on HAART) in this study (Table 1).

Primary vaginal cancer (PCV) is an uncommon gynaecological malignanet disease [23], arising solely from the vagina without cervical or vulval involvement, It affects women over the age of 60 years and has a poor prognosis. In this study, 57% of the vaginal cancers (HSIL) were PCR. About 43–50% of PCR samples demonstrates DNA for HPV, especially that of HPV 18 and 16 (63%) [24–26]. Antibodies for HPV were detected in 28% of the participants in this study. This figure is lower than that reported by Hellman et al. [17] (which range from 43 to 81%) following an intercontinental analysis of HPV infection. According to Brunner et al. [27] and Alonso et al. [28] patients who are positive for HPV infection have better prognosis irrespective of their age and stage of the tumour. This is because, despite the increased proliferation index of HPV infected tumours, they are less genetically complex [29], susceptible to radiotherapy [30,31] and have better prognosis [32]. This study showed that there were more chronic infections (indicated by positive IgG levels) when compared with current or reactivation (indicated by positive levels of IgM). A direct correlation between ASCUS and HPV positivity was observed in this study. This suggests that HPV positivity might be responsible for many premalignant lesions. A combination of HPV positivity and genetic factor could lead to
Table 4
Frequency pattern of EBV, HIV, HPV and HSV co-infections in neoplastic and non-neoplastic vaginal smears.

<table>
<thead>
<tr>
<th>Vaginal Smears</th>
<th>No. of Pts</th>
<th>HIV + HSV2</th>
<th>HSIL + HPV</th>
<th>HSIL + EBV</th>
<th>HIV + HPV+ HSV2</th>
<th>HIV + HPV+ EBV</th>
<th>HPV + HSV2+ EBV</th>
<th>HPV + HIV+ EBV+ HSV2</th>
<th>No of confections</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSIL</td>
<td>7</td>
<td>1 (14.3)</td>
<td>0</td>
<td>1 (14.3)</td>
<td>0</td>
<td>0</td>
<td>4 (57.1)</td>
<td>1 (14.3)</td>
<td>7 (100)</td>
</tr>
<tr>
<td>LSIL</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>3 (33.3)</td>
<td>1 (11.1)</td>
<td>0</td>
<td>4 (44.4)</td>
<td>0</td>
<td>8 (88.9)</td>
</tr>
<tr>
<td>ASCUS</td>
<td>8</td>
<td>0</td>
<td>2 (25.0)</td>
<td>2 (25.0)</td>
<td>0</td>
<td>0</td>
<td>1 (12.5)</td>
<td>3 (37.5)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Positive Smears</td>
<td>25</td>
<td>1 (4)</td>
<td>2 (8)</td>
<td>6 (24)</td>
<td>1 (4)</td>
<td>0</td>
<td>9 (36)</td>
<td>4 (16%)</td>
<td>24 (96.0)</td>
</tr>
<tr>
<td>Negative Smears</td>
<td>149</td>
<td>3 (2.1)</td>
<td>1 (0.8)</td>
<td>4 (2.7)</td>
<td>2 (0.8)</td>
<td>1 (0.8)</td>
<td>9 (6.0)</td>
<td>5 (3.4%)</td>
<td>25 (16.8)</td>
</tr>
<tr>
<td>Total</td>
<td>173</td>
<td>4 (2.3)</td>
<td>3 (1.7)</td>
<td>10 (5.8)</td>
<td>1 (0.6)</td>
<td>1 (0.6)</td>
<td>2 (1.2)</td>
<td>18 (10.4)</td>
<td>9 (5.2)</td>
</tr>
</tbody>
</table>

Figs. 1–4. Fig. 1 is a photomicrograph of vaginal smear showing superficial, intermediate and deep cells without any morphological change. Stained by Papanicolaou technique <400. Fig. 2 is a photomicrograph of vaginal smear depicting ASCUS with evidence of moderate increase in nuclear size and perinuclear halo (marked by arrows). Stained by Papanicolaou technique >100. Fig. 3 above is a photomicrograph of a vaginal smear depicting LSIL with evidence of hyperchromatic nucleus (marked by black arrow), increased nuclear-cytoplasmic ratio (shown by blue arrow) and numerous polymorphonuclear neutrophils and dirty orange background. Stained by Papanicolaou technique >400. Fig. 4 above is a photomicrograph of a vaginal smear depicting HSIL with evidence of hyperchromatic nuclei (marked by black arrows), cytoplasmic elongation and keratinization (characteristic of squamous cell carcinoma; marked by blue arrow) and numerous polymorphonuclear neutrophil. Stained by Papanicolaou technique >400.

the progression of premalignant lesion to malignancy. Hellman et al. [17] reported that small tumours had increased proliferation index (higher Ki67 expression) than large tumours. This report agrees well with our findings which showed higher Ki67 and SCCA1 expression in LSIL compared with HSIL. We also observed more ASCUS (Fig. 2) and LSIL (Fig. 3) in this study compared with the number of HSILs (Fig. 4). These premalignant lesions may progress to HSIL when there are more viral co-infection, depressed immunity, accumulation of more mutations and defective apoptotic mechanisms.

High mitotic activity biomarker, ki67, negatively correlates with a severe tumor grade [17,33] and early disease recurrence. This is in line with our result as serum levels of ki67 and SCCA1 were higher in LSIL than in HSIL. However, the levels of ki67 and SCCA1 in those positive for HSIL was higher than in those negative for intraepithelial lesion (NIL). In our study, 3 cases (12%) of the vaginal lesions were negative for SCCA1 while 2 out of the three cases had higher levels for Ki67 serum levels and were negative for HPV antibodies. This finding is in line with the findings of Schmilovitz-Weiss et al. [18], who reported that SCCA positivity is associated with better prognosis while SCCA negativity is related to increased Ki67 score and poor prognosis. Brunner et al. [27] and Alonso et al. [28] also observed comparable results in hepatocellular carcinoma. Conversely, 7 cases (28%) of vaginal lesions in this study were negative for ki67 while out of the 7 cases, 6 (86%) had higher serum levels of SCCA1. Three 3 cases out of the 7 cases (43%) were positive for HPV antibodies while 4 cases (57%) out of the 7 cases which were negative for HPV antibodies had high SCCA levels. The latter 4 cases may have very poor prognosis since the role of serpins in dysplastic cells suggest that SCCA secretion makes malignant cells unsusceptible to antitumorogenic mechanisms by cell death inhibition [34]. Additionally, our study shows that the sensitivity rate of Ki67 increases
with the stage of the lesion (Table 1), hence Ki67 could be used as a tool for grading vaginal lesions. This is in line with the study carried out by Baak and Kruse [16] and Hellman et al. [17] on the cervix and vagina biopsies, respectively.

This study recorded a lower prevalence of HSV-2 (63%) as against the prevalence of 87% reported by Agabi et al. [35] among patients attending STD Clinic in Jos (Table 3). The difference in prevalence may probably be due to the difference in HIV prevalence between the Plateau and Ogun States. With the explosion of gynaecological studies, it is increasingly evident that HSV-2 facilitates HIV transmission [36,37]. Antiretroviral therapy decreases HSV prevalence and transmission along with decreased HIV viral load [38]. The latter is in consonance with this study as HIV negative participants (who were not on HAART) had more HSV-2 IgG and IgM antibodies positivity compared with HIV seropositive participants (who were on HAART). However, the fact that HSV-2 infection correlates with viral cytopathic effect (Table 3) suggests that HSV-2 plays critical part in the manifestation of vaginal lesions than previously thought.

Epstein-Bar virus (EBV), after primary infection, persist for life in 90–95% of the cases and has been linked to some malignancy including lymphoma and gastric carcinoma. Epstein-Bar Virus-induced malignancy is unique in that its genes are oncogenic even in latent form [39]. In this study, 27% of the participants were positive for EBV (IgG and IgM) antibodies (Table 3). However, only 76% of those who had vaginal lesions were positive for EBV antibodies. This suggests that EBV may have played a critical role in the development of vaginal lesion. Furthermore, HSV and EBV co-infection was only observed among HIV seropositive participants (Table 4). This suggests that HIV infection, which compromises the body’s immunity, favours the co-existence of HSV and EBV. Our study also revealed lower prevalence of 5.8% for HSV and EBV co-infection among those positive for vaginal lesions against the prevalence of 30% reported by Rodrigues et al. [11] in Brazil. The synergistic role of EBV infection in cancer has been reported in genital warts, perianal intraepithelium and severe intraepithelial lesions [14]. Contrary to this, EBV co-infection was observed among participants negative for vaginal lesions. It is important to note that the contribution of EBV to carcinogenic activities varies with the type of latent protein (LMP1, LMP2, EBNA1 or EBNA2) expressed. Among other latent EBV proteins, only LMP1 has oncogenic activity [39]. Thus, those who were infected with EBV but negative for intraepithelial lesion may have been negative for the LMP1 protein.

Co-infection with HPV and EBV has been implicated in anal [14,40] and pharyngeal intraepithelial neoplasia [41]; however this study revealed only 1 case of such co-infection in a participant who was negative for vaginal lesion. In this study, 9 participants were infected with all the investigated viruses; 4 were positive for vaginal lesions while 5 were negative for vaginal lesions (Table 4). This is higher than that of Guimaraes et al. [14], who recorded only one case of such co-infection in their study. Furthermore, 16% of those who were positive for vaginal lesions and seropositive for the four viruses had average age of 38.3 years and average age at sex debut of 21.5 years while 3% of those who were negative for vaginal lesions and seropositive for all four viruses had average age of 49 years and average age at sex debut of 18.8 years. Both groups co-infected with the four viruses, irrespective of their vaginal smear status (positive or negative), expressed similar pattern of positivity for IgG and IgM antibodies. The reason for the negative smears among those who were seropositive for the four virus, despite their higher age in life and lower age at sex debut, is still unclear. However, it could be that the latter were negative for high risk HPV and EBV LMP1 protein. Lastly, the pattern of Ki67 and SCCA1 expression in the vaginal lesions may have been influenced by the proliferative index of CD4 T cells. The pattern of ki67 and CD4 cell expression correlates well with the reports of Li et al. [42] which showed concomitant increase in Ki67 and CD4 T cells following administration of influenza and tetanus toxoid vaccines. This suggests that targeting CD4 T cells expressing Ki67 specific for EBV, HPV and HSV in virus-related malignancy could induce remission and also prevent the progression of premalignant lesion to cancer.

5. Conclusion

This study suggests that there is an increased prevalence of vaginal lesions and viral cytopathic effects among HIV seropositive participants when compared with seronegative participants. It also revealed that early sexual intercourse, viral co-infection and low CD4 cells are risk factors for vaginal lesions. Our study also suggests that SCCA1 and Ki67 could be used as diagnostic and prognostic biomarkers for vaginal lesions. More importantly, patients who are seropositive for all four viruses should be further investigated for high-risk HPVs and LMP1 protein, and closely monitored for early signs of malignancy.

Conflicts of interest

No conflict of interest was declared by the authors.

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