Correlation of P53 and Bcl-2 With the Presence of Hpv-16 in Sudanese Female Patients Diagnosed With Cervical Pre-cancerous and Squamous Cell Carcinoma of The Cervix.

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Research note

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Abstract

Objectives

The persistence of high-risk Human Papilloma virus-16 (HPV-16) is considered as a predisposing factor for the development of the cervical carcinoma and the prognosis of the disease. However, other multifactorial risk factors including cell proliferation and apoptosis markers were also studied and linked to the development and progression of cervical carcinoma. This study is aiming at investigating the association between the presence of HPV-16 and the expression of p53 and Bcl-2 in benign and malignant cervical lesions using immunohistochemical staining methods.

Results

A total of 150 cases with different cervical lesions were studied, their age ranges from 34 to 67 years (mean 49.5 ± 6.9). Out of the 150 cases; 97 (64.7%) were malignant, 37 (24.7%) were pre-cancerous and 16 (10.7%) were benign lesions. The association between the expression of Bcl-2 and p53, the different histopathological diagnosis, and the type of lesion was statistically significant (p value < 0.0001) Furthermore the statistical analysis of Bcl-2 expression revealed a significant correlation with the cancer grade (p value = 0.018). Nevertheless, p53 expression showed no association with the cancer grade. Regarding the correlation between the presence of HPV-16 and the markers the results were found to be statistically significant.

Introduction:

Cervical cancer is a type of cancer that arises from the uterine cervix. It is also considered as the fourth most common type of cancer among females [1–4]. Although different hypotheses were investigated to better understand the induction and the progression of cervical cancer, the exact mechanism was not fully explained [5]. Multiple factors were proven to contribute in the induction of cervical carcinoma including Human Papilloma virus (HPV) [6–8] and the genetic alterations and mutations in the human genome [9–11]. HPV is considered as an important etiological agent in cervical cancer development since the mechanism of HPV-induced cancer is related to the deactivation of tumor suppressor genes by the viral oncoproteins [12]. However, in spite of the role of HPV; many studies also considered mutation in cell proliferation genes in addition to apoptotic genes such as p53 and B cell Lymphoma-2 (Bcl-2) also play a role in the development of cervical cancer [13, 14]. The p53 plays an important role in determining the fate of cells following exposure to certain stimuli that damage the DNA or when the damage is exceeding the repairability of the cells [15–18]. Therefore; cells with a mutated p53 are unable to repair and the net results is the appearance of malignant cells [17]. Whereas, Bcl-2 gene blocks the apoptosis of cells and thus inhibits the death of the cells [18–20]. The expression of the Bcl-2 gene was detected in many solid tumors including Ovarian carcinoma [21, 22]; breast [23]; prostate [24] and colorectal cancers [25]. Therefore, this report investigates the expression of p53 and Bcl-2 in different precancerous and

cancerous lesions of the uterine cervix and correlates the expression of these markers with the clinical staging and the presence of the high-risk HPV-16.

Materials And Methods:

This is a descriptive, hospital- based study conducted at different histopathology Laboratory, during a period from December 2017 till May 2019 in Khartoum State, Sudan. 150 formalin-fixed paraffin embedded blocks (FFPB) were collected from female patients diagnosed with cervical abnormalities. Clinicopathological features, such differentiation grade, and stage, were obtained.

Preparation of the slide sections:

From each FFPB four sections were cutted using Leica rotary microtome; three sections were cut with a thickness of 3 μ and single section was cut at a thickness of 20 μ the latter one was transferred to 1.5 eppendorf tube prior to DNA extraction. From the three 3 μ-tissue sections one was stained using hematoxylin and eosin staining technique, one was stained using p53 primary antibody, and the third one was stained with Bcl-2 primary antibody.

Immunohistochemistry (IHC) slide preparation:

Prior to the IHC staining, two slides were mounted onto OptiPlus™ positively – charged microscopy slides (BioGenex lot No. XT0020115I, Fremont; CA; USA). To retrieve the antigenic sites from tissue sections the slides were subjected to treatment with citrate buffer at 96^{o} C for 15 minutes in a waterbath. Then, the tissue sections were rinsed first in distilled water and then with Tris buffer saline (TBS), followed by treatment with peroxidase block (3% hydrogen peroxide in methyl alcohol) for 15 minutes to quench endogenous peroxidase activity. These slides were then placed in a humid chamber. Then the slides were drained and rinsed in two successive changes of Tris buffer (wash buffer), for 5 minutes for each. Nonspecific protein–protein interactions were blocked by treating and incubating the tissue sections with the power block (casein in phosphate buffered saline) for 10 minutes in a humid chamber. Then, the remaining solution was drained from the slides. The sections were then incubated in p53 and Bcl-2 primary antibodies (Abcam 154036 and ab59348 respectively) at 4 °C overnight in the humid chamber. After that, the staining was performed according to manufacturer protocol. A section from a known colon cancer tissue block was used as a positive control and PBS stained tissue block (instead of the primary antibodies steps for both markers) was used as a negative control.

Assessment of immunoreactivity of p53 and Bcl-2:

p53 expression was confined to the nucleus; whereas Bcl-2 expression was confined to the cytoplasm. The scoring system for p53 and Bcl-2 expression was based on semi quantitative method on which we were counting five fields and from each field one hundred cells were counted and the mean percentage positivity was calculated. Expression of p53, and Bcl-2 was considered as positive if more than 5% of cells showed positivity and if less than 5% positivity were rendered as a negative expression.

DNA extraction and detection of HPV-16 from paraffin embedded tissue Blocks

After cutting the tissue block with 20 μ thickness it was directly transfer to a sterile 1.5 eppendorf tube; then for each tissue section we added 1 ml of xylene and incubated for one hour, and with the aid of heating to accelerate the removal of the wax. Then, tissues sections were adjusted to genomic DNA using QIAamp DNA Mini Kit (Qiagen, Germany).

Following the DNA extraction, DNA templates were prepared for the detection of HPV-16 using the polymerase chain reaction (PCR) technique using the following primers; forward: 5'TTT TGG GTT ACA CAT TTA CAA G'3 and reverse: 5'TGT CTG CTT TTA TAC TAA CCG'3. The PCR amplification reaction was adjusted to initial denaturation stage at 94 °C for 5 minutes, then 35 cycles of 94 °C for 1 minute, and annealing at 55 °C for 1 minute, followed by an extension at 72 °C for 1 minute and a final extension stage at 72 °C for 10 minutes. The PCR products were then visualized in a 2% agarose gel to visualize the expected band size of 119 bp.

Statistical analysis:

The statistical analysis was done using the Statistical Package for Social Sciences (SPSS, v20.0). The Chi-Squared test was used to compare the frequencies of the categorical variables. A value of $p < 0.05$ was considered statistically significant.

Results:

Analysis based on age groups:

The study included 150 female patients diagnosed with cervical lesions, their age ranges from 34 to 67 years. The mean age of patients was 49.5 ± 6.9 years. Most of the study participants 82 (54.7%) were in the age group of 41 to 50 years, followed by the age group of 51 to 60 years; 48 (32.0%). Age groups of 31 to 40 years and 61 to 70 years were the least age groups represented by the study participants; 12 (8.0%) and 8 (5.3%), respectively.

The most frequent tumor type among the study participants was cervical cancer; 97 (64.7%), followed by pre-cancerous; 37 (24.7%), and benign; 16 (10.7%). The pre-cancerous lesions included 20 (13.3%) mild dysplasia, and 17 (11.3%) severe dysplasia, while cancerous lesions included 21 (14.0%) poorly differentiated squamous cell carcinoma (SCC), 44 (29.3%) moderately differentiated SCC, and 32 (21.3%) well differentiated SCC. The benign lesions only included 16 (10.7%) cervicitis. Based on the histopathological diagnosis, among the age group 51 to 60 years, 21 (65.6%) and 20 (45.5%) were diagnosed with well differentiated SCC and moderately differentiated SCC, respectively. A statistically significant difference was seen between the different histopathological diagnosis's classifications among the different age groups, (P value 0.000) (Additional file 1).

Comparing the cancer stage against the histopathological diagnosis

The distribution of cancer stages among those diagnosed with SCC showed that stages II and III cancer were the most frequent stages. However, no statistically significant difference was seen; P value 0.755. Stage II was most frequently 16/33 (48.5%) among the age group 51 to 60 years. Meanwhile, stage III was most frequent among 16/32 (50.0%) among 41 to 50 years age group. (Additional file 1)

Immunohistochemical expression of Bcl-2 and p53

The immunohistochemical expression of Bcl-2 and p53 among the different types of tumors showed that Bcl-2 and p53 were only expressed among pre-cancerous and cervical cancer patients (Fig. 1).

The expression of Bcl-2 among pre-cancerous lesions was relatively similar to the expression among cervical cancer lesions. Whereas, p53 was relatively over-expressed among cervical cancer lesions compared to the pre-cancerous lesions. The details of immunohistochemical expression of Bcl-2 and p53 among the different histopathological diagnosis and cancer staging for lesions diagnosed as SCC are illustrated in Table 1.

Table 1
Relationship of different variables in study population with p53 and Bcl-2 expression.

	BcL2			P53		
	Negative	Positive	P value	Negative	Positive	P value
Tumor type						
Benign	16 (13.9%)	0 (0.0%)	0.000	16 (22.2%)	0 (0.0%)	0.000
Pre-cancerous	19 (16.5%)	18 (51.4%)		16 (22.2%)	21 (26.9%)	
Cervical Cancer	80 (69.6%)	17 (48.6%)		40 (55.6%)	57 (73.1%)	
Total	115 (76.7%)	35 (23.3%)		72 (48.0%)	78 (52.0%)	
Histopathology diagnosis						
Cervicitis	16 (13.9%)	0 (0.0%)	0.000	16 (22.2%)	0 (0.0%)	0.000
Well differentiated SCC	27 (23.5%)	5 (14.3%)		12 (16.7%)	20 (25.6%)	
Moderately differentiated SCC	37 (32.2%)	7 (20.0%)		18 (25.0%)	26 (33.3%)	
Poorly differentiated SCC	16 (13.9%)	5 (14.3%)		10 (13.9%)	11 (14.1%)	
Mild dysplasia	19 (16.5%)	1 (2.9%)		16 (22.2%)	4 (5.1%)	
Severe dysplasia	0 (0.0%)	17 (48.6%)		0 (0.0%)	17 (21.8%)	
Total	115 (76.7%)	35 (23.3%)		72 (48.0%)	78 (52.0%)	
Cancer grade						
Grade I	16 (20.0%)	2 (11.8%)	0.018	8 (20.0%)	10 (17.5%)	0.293
Grade II	23 (28.8%)	10 (58.8%)		11 (27.5%)	22 (38.6%)	
Grade III	31 (38.8%)	1 (5.9%)		17 (42.5%)	15 (26.3%)	
Grade IV	10 (12.5%)	4 (23.5%)		4 (10.0%)	10 (17.5%)	

	BcL2			P53		
	Negative	Positive	P value	Negative	Positive	P value
Total	80 (82.5%)	17 (17.5%)		40 (41.2%)	57 (58.8%)	

Correlation of HPV-16 with the immunohistochemical expression of Bcl-2 and p53

HPV-16 was detected among 29/150 (19.3%) patients, of them 11 (37.9%) were pre-cancerous lesions and 18 (62.1%) cancerous lesions. Among the pre-cancerous; 3/29 (10.3%) were mild dysplasia and 8/29 (27.6%) were severe dysplasia. Whereas among the cervical cancer lesions; 8 (27.6%) moderately differentiated SCC, and 5 (17.2%) were well- and poorly differentiated SCC, each. The prevalence of HPV-16 among the different types of lesions was noted to be statistically significant, P value 0.023. Also, the expression of Bcl-2 and p53 was positively correlated with the presence of HPV-16, Pearson's correlations were [r = 0.470, P value 0.000] and [r = 0.728, P value 0.000], for Bcl-2 and p53, respectively (Table 2).

Table 2
Correlation between the presence and the absences of the HPV-16 and expression of BcL-2 and p53.

	HPV16		Total	Pearson's *r*	P value
	Negative	Positive			
Tumor type					
Benign	16 (100%)	0 (0.0%)	16 (10.7%)	0.058	0.48
Pre-cancerous	26 (70.3%)	11 (29.7%)	37 (24.7%)		
Cervical Cancer	79 (81.4%)	18 (18.6%)	97 (64.7%)		
Histopathology diagnosis					
Cervicitis	16 (100%)	0 (0.0%)	16 (10.7%)	0.234	0.004
well differentiated SCC	27 (84.4%)	5 (15.6%)	32 (21.3%)		
Moderately differentiated SCC	36 (81.8%)	8 (18.2%)	44 (29.3%)		
Poorly differentiated SCC	16 (76.2%)	5 (23.8%)	21 (14.0%)		
Mild dysplasia	17 (85.0%)	3 (15.0%)	20 (13.3%)		
Severe dysplasia	9 (52.9%)	8 (47.1%)	17 (11.3%)		
Cancer stage					
Grade I	16 (88.9%)	2 (11.1%)	18 (12.0%)	-0.022	0.83
Grade II	22 (66.7%)	11 (33.3%)	33 (22.0%)		
Grade III	31 (96.9%)	1 (3.1%)	32 (21.3%)		
Grade IV	10 (71.4%)	4 (28.6%)	14 (9.3%)		
P53 expression					
Negative	72 (100%)	0 (0.0%)	72 (48.0%)	0.470	0.0001
Positive	49 (62.8%)	29 (37.2%)	78 (52.0%)		
BCL2 expression					
Negative	111 (96.5%)	4 (3.5%)	115 (76.7%)	0.728	0.0001
Positive	10 (28.6%)	25 (71.4%)	35 (23.3%)		

Discussion:

In this study we investigated the correlation of HPV-16 and the expression of p53 and Bcl-2 in different subsets of cervical lesions. The expression of p53 and Bcl-2 was confined to pre-cancerous and

cancerous lesions. These findings go beyond previous reports, showing that, p53 and Bcl-2 expression is highly associated with cervical cancer [27–29], additionally, some studies of these markers in other type of cancer gave similar results [29, 30], all together, these findings highlight the role of these markers in cervical cancer progression and prognosis, however, the data about Bcl-2 expression in cancer is highly variable, as noted in some studies were the strong role of this marker was reported especially with hematopoietic cancers [31, 32], while many studies reported that Bcl-2 expression had no association [29, 33]. However, in line with our results, previous studies on the expression of Bcl-2 among pre-cancerous and cancerous lesions was almost similar, while p53 was positively associated with cervical cancer [28, 34, 35]. These findings indicate the possible role of p53 to differentiate between borderline and malignant tumor. However, based on our findings, applying these two markers give more valuable information about the severity of pre-cancerous lesions since both of them were exclusively expressed among cases of severe dysplasia.

In the current study HPV-16 was not detected among cervicitis cases, however exclusively detected among pre-cancerous and cancerous conditions, moreover most of these cases were aged more than 41 years which is constant with Zhang *et al.*, in a research done among Chinese women [35], and this fact is in disagreement with many studies reporting that most women affected by cervical cancer were among those at their 3rd decade of their life [1, 36], however, the relationship between HPV viruses and risk of cervical cancer depends on many factors, and need to be studied in large series of samples. Regarding p53 and Bcl-2 expression a high positive correlation with HPV-16 was reported by our study, which is in accordance with the previous fact about high risk of this HPV-16 subtype which can produce several type of proteins that can be integrated with p53 and in turn lead to enhancement of cell life span [38, 39], in addition to this direct effect inactivation and accumulation of this impaired p53 protein can induce Bcl-2 up-regulation [32], Conversely with our results Shukla *et al.*, found that expression of Bcl-2 and p53 have no significant association with HPV-16 [1], and this may attribute to the complexity of viral oncogenic proteins which can influence p53 and Bcl-2 by different manners depending on a dominant variant of viral oncoprotein, and some of these proteins can lead to complete loss for p53 [32]. Nevertheless, the relationship between p53 and Bcl-2 is highly complex and according to some studies p53 can block Bcl-2 expression in presence of some molecules, this implies that the role of these markers in cervical cancer is highly affected by HPV-16 related proteins [39, 40].

Conclusion:

Co-expression of p53 and Bcl-2 among pre-cancerous and cancerous lesions of cervical cancer is significantly associated with HPV-16 infection. Furthermore, the expression of these markers was associated with the histopathological diagnosis of cervical lesions.

Limitations:

- Lack of follow-up data about the patients and the other HPV strains could help in predicting precancerous lesions behavior as well as the progression of malignant lesions.

Abbreviations

DNA
Deoxy ribonucleic acid
HPV
Human Papilloma virus
IHC
Immunohistochemistry
PCR
Polymerase chain reaction
SCC
Squamous cell carcinoma

Declarations

Ethics approval and consent to participate

The study ethics approval and consent to participate were obtained by the ethics review board of the Faculty of Medical Laboratory Sciences, University of Khartoum. Informed consent was obtained from each participant prior to enrollment using writing and verbal informed consent.

Consent to publish

Not Applicable.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Not Applicable.

Authors' contributions

AMM and EES conceived and designed the study; AMM, EES performed the study and provided the reagents; ETA, NSM, IRS, MSM, AMM and EES analyzed the data; ETA, NSM, IRS, MSM, AMM, MF and EES wrote the manuscript. ETA, NSM, IRS, MF and EES revised the manuscript. All authors read and approved the final manuscript.

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Figures

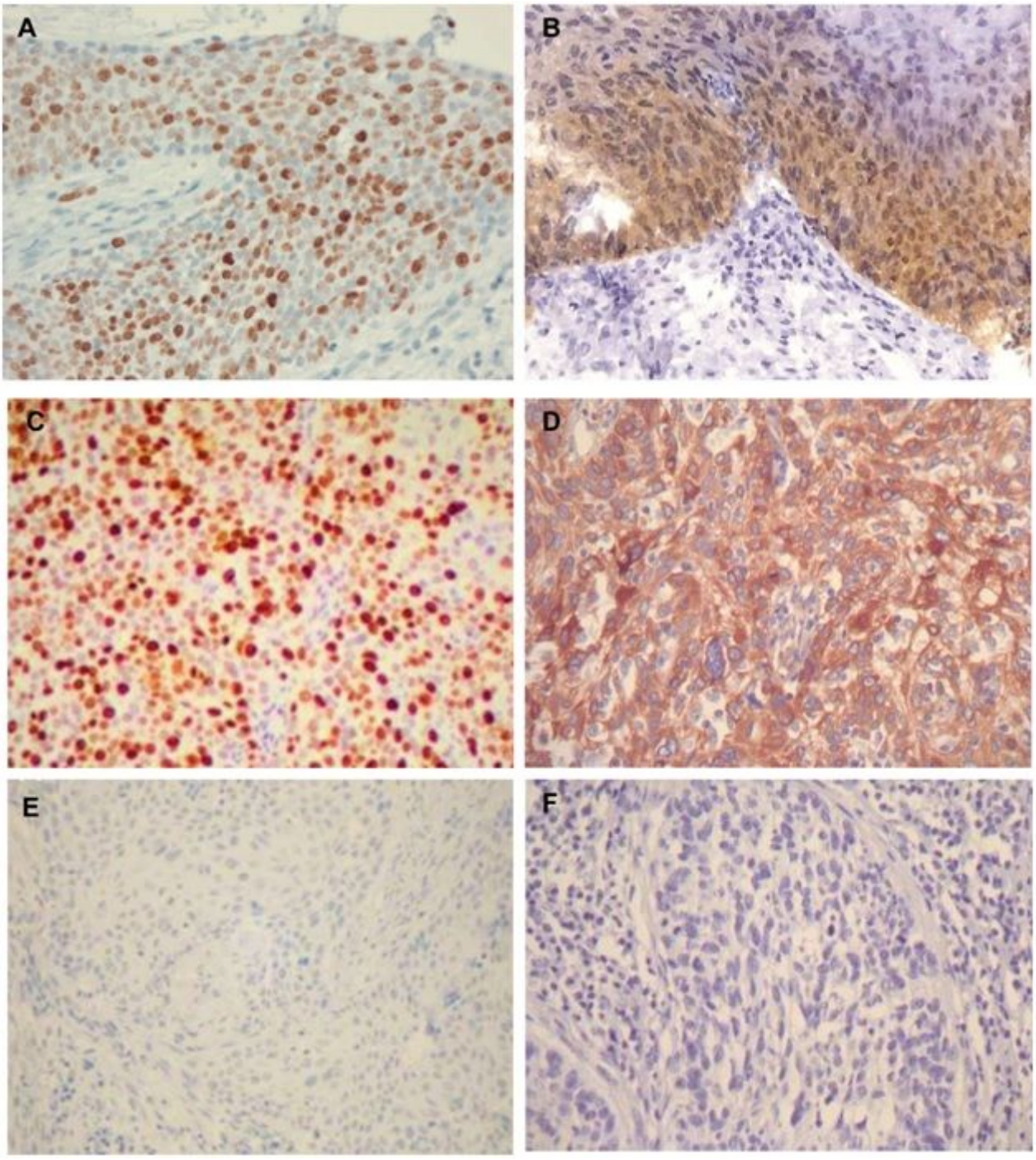

Figure 1

Representative images for Immunohistochemical expression of p53 and Bcl-2 in cervical lesions. Sections stained with Mayer`s hematoxylin-DAP peroxidase. Microscopical magnification X20; A: severe dysplasia positive for p53, B: severe dysplasia positive for Bcl-2, C: Squamous cell carcinoma positive for p53, D: Squamous cell carcinoma positive for Bcl-2, E: squamous cell carcinoma negative for p53, F: squamous cell carcinoma negative for Bcl-2.

Supplementary Files

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- Additionalfile1.docx