ORIGINAL ARTICLE

Study of CD31 IHC expression in dysplastic and malignant lesions of the cervix and its correlation to IHC expression of HPV (16E6 + 18E6)

Mohanad Mundher Abdulghani[1], Alaa Qasim Yahya[1], Mohammed N. Hilmi[1], Ahmad Fawzi Hussain[2]*

[1]Department of Pathology and Forensic Medicine, Al Kindy College of Medicine, University of Baghdad-00964, Iraq, [2]Department of Gynecology and Obstetrics, Medical Faculty, Justus-Liebig-University Giessen, Aulweg-128, 35392 Giessen, Germany

Abstract

Background: Angiogenesis is necessary for invasion and metastasis of cervical carcinoma and is a crucial stage in controlling the course of malignancy. Microvasculature may be quantified by calculating Mean Microvessel Density (MVD) using CD31 Immunohistochemical (IHC) labeling of endothelial cells. E6 and E7 oncogenes of Human Papillomavirus (HPV) will inactivate p53 and pRb, respectively. This has been discovered to encourage angiogenesis by up-regulating vascular endothelial growth factor and down-regulating thrombospondin-1 during the early stages of carcinogenesis. *Aim and Objectives:* To assess the IHC expression of CD31 in cervical dysplasia and carcinomas and link these expressions with the IHC expression of HPV (16E6 + 18E6) and specific clinicopathological criteria, such as stage and grade of cervical carcinoma. *Material and Methods:* In a cross-sectional investigation, 52 cervical tissue samples were formalin-fixed and paraffin-embedded. On hematoxylin and eosin slides, the histological diagnoses were obtained. Samples were then stained immunohistochemically with monoclonal mouse antibodies, including anti-CD31 antibody and Anti-HPV (16E6 + 18E8) antibody. *Results:* Mean MVD rose significantly with increasing cervical lesion severity. In 78.1% of patients with invasive cervical carcinomas, HPV expression was detected, compared to 41.7% of cases with high-grade squamous intra-epithelial lesion and 37.5% of cases with low-grade squamous intra-epithelial lesion. *Conclusion:* HPV plays a crucial role in malignant transformation of the cervix, with a substantial positive association between a rise in MVD and expression of high-risk HPV types (16 and 18) in malignant and premalignant instances.

Keywords: CD31 IHC, microvessel density, cervical carcinoma, human papilloma virus, angiogenesis

Introduction

Cervical dysplasia, or Squamous Intraepithelial Lesions (SILs), is a precursor to the cervical cancer. The condition is the result of chronic infection of cervical tissue by Human Papillomavirus (HPV). HPV 16 is the most common type that is responsible for 50% of cervical cancer [1]. Cervical cancer ranks as the fourth most common cause of cancer incidence and mortality in women worldwide [2]. However, according to reports from the Iraqi Cancer Registry in 2015, cervical cancer is no longer among the top 10 female cancers in Iraq [3]. It takes a median time of 23.5 years for Cervical Intraepithelial Neoplasia (CIN) II-III to progress to cancer, allowing ample time for early detection and potential preventive treatment of cancer or precancerous lesions [4]. Angiogenesis is typically initiated from the capillaries and plays an important role in tumor growth, maintenance, and metastasis. Stromal blood vessels are an essential factor for epithelial growth. Angiogenesis will be necessary for invasive tumor growth and metastasis and is a crucial step in controlling the progression of cancer

[5]. Angiogenesis cannot be directly evaluated, but microvasculature can be quantified by measuring Mean Microvessel Density (MVD). This method involves Immunohistochemical (IHC) staining of capillary endothelial cells with monoclonal antibodies [6].

In order to measure MVD, IHC staining is necessary. This method often involves using antibodies against the endothelial cell markers CD31, CD34, and CD105, which have been widely employed in past cancer tissue research. [7]. HPV 16/18 are the two most common genotypes in approximately 70% of invasive cervical cancer cases [8]. Several steps in the viral life cycle and replication rely on the genes encoded by the HPV genome. In high-risk HPV, the two most significant genes are E6 and E7, which are also called the main viral oncogenes. Although p53 and pRb are the primary targets of E6 and E7, respectively, these viral oncogenes were named for the cell targets and consequences of their dysregulation [9] and it is well known that loss or inactivation of p53 promotes hypoxia-induced angiogenesis [10]. The purpose of this work is to examine IHC expression of CD31 in cervical dysplasia and carcinomas and to link these expressions with IHC expression of HPV (16E6 + 18E6) and specific clinicopathological criteria, such as grade of SIL and carcinoma and stage of cervical carcinoma.

Material and Methods

Fifty-two cervical tissue samples were included in this cross-sectional analysis; 8 were diagnosed with Low-grade SIL (LSIL) and 12 with High-grade SIL (HSIL), and 32 cases of invasive carcinoma, including 6 cases of adenocarcinoma and 26 cases of squamous carcinoma, were selected from the archives of Al-Ellewea teaching hospital for gynecology and obstetrics in Baghdad from January 2018 to December 2020. On hematoxylin and eosin-stained slides, an expert pathologist made the first histological diagnosis. Patients' demographics, histological types of their cervical carcinomas and dysplasia, and their pathological stages according to International Federation of Gynecology and Obstetrics (FIGO) were collected from their admission case sheets and pathology reports. IHC expression was scored according to the following semi quantitative scale:

1. The absence of staining indicated negative staining.
2. 25% indicated weakly positive staining.
3. 25% to 50% indicated moderate positive staining.
4. >50% suggested significant immunostaining [11].

Statistical analysis

Categorical variables were reported as numbers and percentages; whereas continuous variables were expressed as mean and Standard Error of Mean (SEM). Fisher's exact test and the Pearson Chi square test were used to compare percentages of findings. Relationships between categorical and continuous variables were examined using unpaired t-test and analysis of variance. All calculations were performed using version 18 of Statistical Package for Social Sciences (SPSS) (SPSS Inc., Chicago, IL, USA), and value of $p< 0.05$ was considered statistically significant.

Results

There was a correlation between age at diagnosis and disease status; older women were diagnosed with invasive cervical cancer. Table 1 describes clinicopathological characteristics of present investigation.

IHC manifestation of CD31

MVD (measured by IHC expression of CD31) increased significantly with increasing cervical lesion severity and cervical cancer stage and grade ($p = 0.001$). However, the comparison of cervical squamous cell carcinoma and cervical adenocarcinoma did not achieve statistical significance ($p = 0.291$) (Table 2).

Expression of HPV (16E6 + 18E6) via IHC

The IHC expression of HPV (16E6 + 18E6) was significantly increased with severity of cervical carcinoma ($p = 0.021$) with minor correlation between HPV (16E6 + 18E6) with cancer stage and grade (Table 3).

Correlation between markers

There was a statistically significant positive connection between IHC expression of HPV (16E6 + 18E6) and IHC expression of MVD (detected by IHC expression of CD31) in investigated patients r = 0468 and $p < 0.001$).

Table 1: Clinicopathological factors for mild and high grade squamous intraepithelial lesions and invasive cervical cancer

Parameters			Values
Age: mean (range ± SEM) years		LSIL	34.25 ± 2.9 24-44
		HSIL	40.00 ± 2.6 28-51
		Invasive cancer	49.91 ± 1.6 38-70
Histopathological diagnosis	Low-grade Squamous Intraepithelial Lesions (LSIL)	8	15.4%
	High-grade Squamous Intraepithelial Lesions (HSLI)	12	23.1%
	Invasive cancer	32	61.5%
Histopathological type of invasive cancer	Adenocarcinoma	6	18.8%
	Squamous cancer	26	81.2%
Stage of invasive cervical cancer	Stage I	5	15.6%
	Stage II	20	62.5%
	Stage III	7	21.9%
	Stage IV	00	0.0%
Grade of invasive cervical cancer	Well differentiated	3	9.4%
	Moderately differentiated	17	53.1%
	Poorly differentiated	12	37.5%

Table 2: Association of microvessel density detected by immunohistochemical expression of CD31 with clinical parameters of cervical carcinoma

Parameters		MVD (Mean ± SEM)	*p*
Histopathological diagnosis	**Low-grade Squamous Intraepithelial Lesions**	3.378 ± 3.192	<0.001
	High-grade Squamous Intraepithelial Lesions	14.302 ± 2.607	
	Invasive cancer	57.855 ± 1.596	
Histopathological type of invasive cancer	**Adenocarcinoma**	53.6036 ± 2.73998	0.299
	Squamous cancer	58.8358 ± 2.27268	
Stage of invasive cervical cancer	**Stage I**	48.919 ± 3.491	<0.001
	Stage II	55.135 ± 1.745	
	Stage III	72.008 ± 2.950	
	Stage IV	00.00	
Grade of invasive cervical cancer	**Well differentiated**	49.550 ± 4.751	<0.001
	Moderately differentiated	52.623 ± 1.996	
	Poorly differentiated	67.342 ± 2.375	

Table 3: Immunohistochemical expression of HPV (16E6 + 18E6) with clinicopathological parameters of dysplastic and invasive cervical carcinoma

Parameters		Positive HPV (16E6 + 18E6)	Negative HPV (16E6 + 18E6)	Total	*p*
Histopathological diagnosis	**Low-grade Squamous Intraepithelial Lesions**	3 (37.5%)	5 (62.5%)	8 (100%)	0.021
	High-grade Squamous Intraepithelial Lesions	5 (41.7%)	7 (58.3%)	12 (100%)	
	Invasive cancer	25 (78.1%)	7 (21.9%)	32 (100%)	
Histopathological type of invasive cancer	**Adenocarcinoma**	5 (83.3%)	1 (16.7%)	6 (100%)	0.732
	Squamous cancer	20 (76.9%)	6 (23.1%)	26 (100%)	
Stage of invasive cervical cancer	**Stage I**	3 (60%)	2 (40%)	5 (100%)	0.538
	stage II	16 (80%)	4 (20%)	20 (100%)	
	Stage III	6 (85.7%)	1 (14.3%)	7 (100%)	
	Stage IV	00	00	00	
Grade of invasive cervical cancer	**Well differentiated**	2 (66.7%)	1 (33.3%)	3 (100%)	0.338
	Moderately differentiated	15 (88.2%)	2 (11.8%)	17 (100%)	
	Poorly differentiated	8 (66.7%)	4 (33.3%)	12 (100%)	

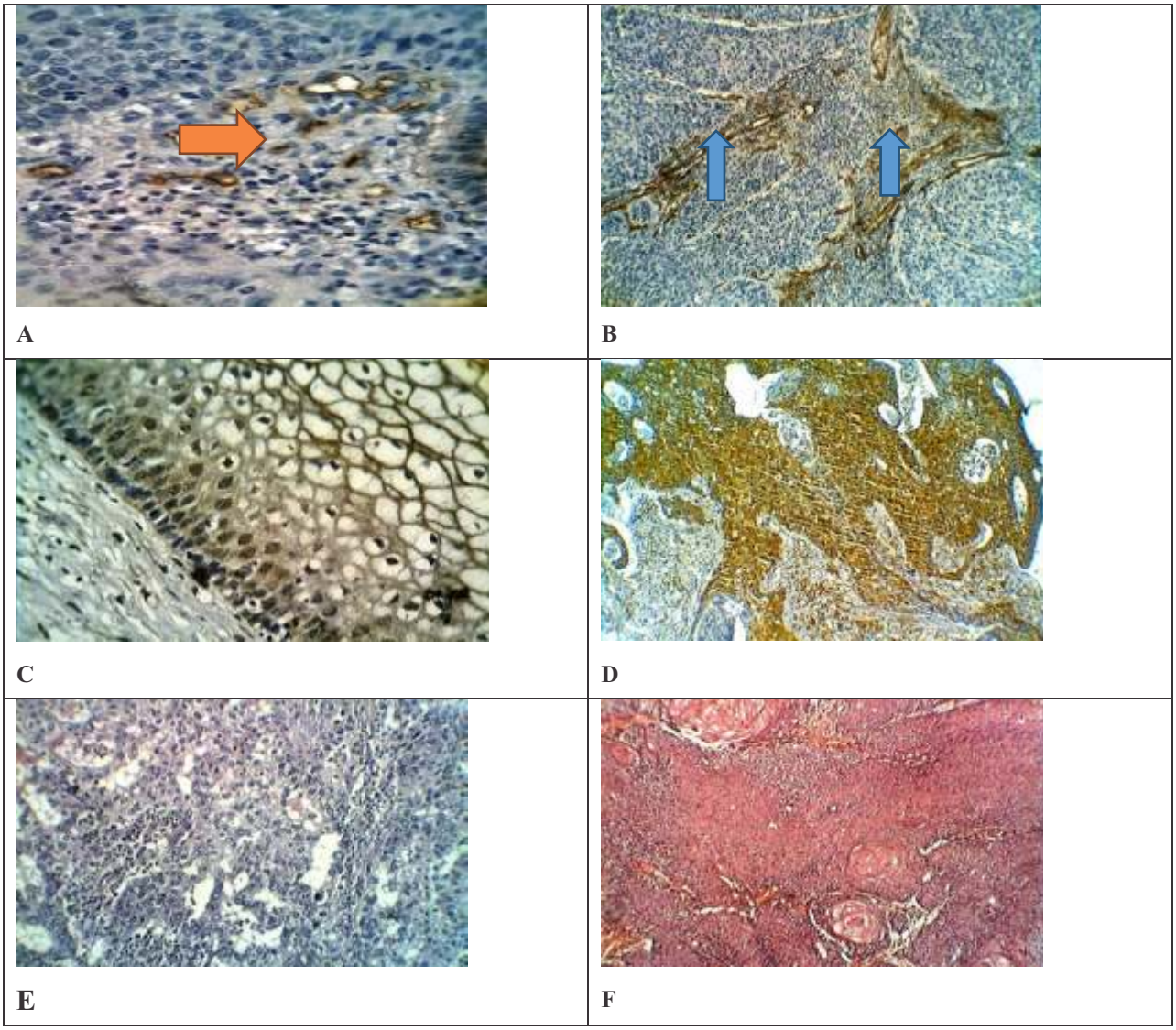

Figure 1A: Microvessel density is somewhat elevated in the connective tissue just below the basement membrane of the dysplastic epithelium, as shown by immunohistochemical staining with an anti-CD31 monoclonal antibody in a high-grade squamous intraepithelial lesion of the uterine cervix (arrow) (40×).

Figure 1B. Immunohistochemical staining of a moderately differentiated uterine cervix squamous cell carcinoma with anti-CD31 monoclonal antibody reveals a considerable rise in intratumoral microvessel density (MVD) and a positive cytoplasmic staining of microvascular endothelial cells (arrows) (10×).

Figure 1C. Positive brown nuclear and cytoplasmic staining with considerable immunostaining (+ +) was seen by immunohistochemistry (IHC) in a low-grade squamous intraepithelial lesion of the uterine cervix using anti-HPV (16E6 + 18E6) monoclonal antibody (40×).

Figure 1D. Positive brown nuclear and cytoplasmic staining with widespread immunostaining (++ +) is shown with immunohistochemical staining of moderately differentiated non-keratinizing squamous cell carcinoma of the uterine cervix using anti-HPV (16E6+ 18E6) monoclonal antibody (10×).

Figure 1E. Moderately differentiated adenocarcinoma showing malignant cells with prominent nucleoli arranged in irregular glands and in sheets, (H&E), (20×).

Figure 1F. Well differentiated squamous cell carcinoma showing irregular nest of malignant squamous cells with keratin pearls, (H&E), (10×).

Discussion

Tumor suppressor gene loss, apoptosis evasion, and angiogenesis overexpression are hallmarks of malignant cells. When it comes to reproductive-aged women, cervix cancer is second only to breast cancer in terms of frequency. In 2012, there were 528,000 new cases worldwide, with 85 percent of those infections occurring in underdeveloped countries. Early detection of cervical diseases can be aided by expanding our understanding of molecular alterations and immunohistochemistry. By incorporating more specific biomarkers into routine diagnosis, we can enhance diagnostic accuracy and reduce the need for unneeded invasive examinations. [12-13].

This study demonstrates that patients with cervical carcinoma are significantly older than those with cervical dysplasia, (Table 1), which is consistent with other Indian and Iraqi studies [14-16] The current series demonstrates that squamous cell carcinoma was the most prevalent form among cervical carcinomas evaluated cases 26 (81.2%), followed by adenocarcinomas instances 6 (18.8%), (Figure 1). This conclusion is consistent with other research conducted by Shukla *et al*., (2014) [14], an Iraqi research conducted by Baythoon *et al*., (2008) [17], and Al-Jewari *et al*., (2007) [18]. In terms of the histological grade of the examined instances of cervical cancer, the majority of cases 17 (53.1%) were moderately differentiated, followed by poorly differentiated cases 12 (37.5%), and just 3 (9.4%) were well differentiated. This conclusion is consistent with prior Iraqi and international research findings [14,17-19].

Regarding FIGO pathology, the majority of studied instances of cervical cancer were stage II. This observation is consistent with the findings of de Oliveira *et al*., (2013) [20], whereas the majority of cases in Xie *et al*., (2020) [21] were FIGO stage I. This discord may be attributable to differences in the effectiveness of cervical cancer screening systems between different countries, including Iraq.

In cancer tissues, CD31 has been found in both the preexisting and newly created vasculatures. Consequently, it continues to be extensively utilized for MVD measurement in human cancer tissues [22]. With increasing cervical lesion severity from LSIL to carcinoma (p = 0.001), present research shows a substantial rise in MVD (detected by IHC expression of CD31), as shown in (Table 2). This is consistent with previous Iraqi studies by Chaloob *et al*., (2016) [23] that used CD 34 and Stepan *et al*., (2012) [24] who used CD105 for assessing MVD, both of whom observed a substantial rise in MVD with increasing severity of cervical lesion from LSIL and HSIL to cancer. The present investigation demonstrates a significantly larger MVD (detected by CD31 IHC expression) in patients with squamous cell carcinomas compared to those with adenocarcinomas (p = 0.01), as shown in (Table 2). This data is consistent with Vieira *et al*., (2004) [25], but Ancuta *et al*., (2010) [19] reported lesser expression in squamous cell carcinoma than in other kinds. This gap may be a result of a specific percentage variation between various forms of cervical carcinoma and the varied methods utilized to measure MVD. Taking into consideration the pathological FIGO stage of cervical cancer, this research demonstrates a substantial increase in MVD with increasing pathological FIGO stage in cervical carcinoma patients (p = 0.012), (Table 2), in cooperation with the

results of Chaloob *et al*., (2016) [23]. MVD of poorly differentiated carcinomas was found to be substantially greater than that of well differentiated carcinoma (p = 0.001), (Table 2). This result is consistent with those of Ancuta *et al*., (2010) and Stepan *et al*., (2012) [19, 24]. The current study found that expression of HPV (16E6 + 18E6) significantly rose with severity of lesions, from LSIL (37.5%) to HSIL (41.7%) to carcinoma (78.1%), (p = 0.021), (Table 3). These findings are analogous to a prior study conducted by Jalilvand *et al*., (2014) and by Fahad *et al*., (2011) in Iraq [26, 27]. When we examined the IHC expression of HPV (16E6 + 18E6) in cervical carcinomas of various stages and grades, we found no significant difference (p = 0.05), (Table 3), consistent with other studies [28, 29]. HPV16 E6 facilitates Vascular Endothelial Growth Factor (VEGF) induced endothelial cell migration, proliferation, and tube formation in vitro and is considered as one of the key regulators of angiogenesis in the pathophysiology of solid tumors [20]. The current results indicate a substantial positive connection (p < 0.05) between MVD and HPV (16E6 + 18E8) in both SILs and cancer patients, consistent with findings by Ancuta *et al*., (2010) [19]. By directly stimulating the VEGF gene's promoter, HPV16-E6 oncoprotein may contribute to tumor angiogenesis. HPV16-E6 oncoprotein inhibits the ubiquitin-mediated degradation of Hypoxia Inducible Factor-1a (HIF-1a), a transcription factor involved in the activation of the VEGF gene promoter in response to hypoxia [30]. In addition, it has been demonstrated that the HPV16-E7 oncoprotein increases the release of VEGF whereas the HPV16-E5 oncoprotein upregulates VEGF expression by activating a variety of signaling pathways [30].

Conclusion

MVD, as shown by the IHC expression of CD31, was sequentially upregulated during the neoplastic progression of cervical neoplasms from SIL to carcinoma. Consequently, it is an important marker for the risk of malignant transformation and tumor progression in cervical lesions and plays a significant role in the progression of cervical neoplasms. Patients with cervical cancer, whether advanced or precancerous, are more likely to express high-risk HPV types 16 and 18, and this is positively correlated with an increase in MVD.

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Author for Correspondence:
Dr. Alaa Qasim Yahya, Department of Pathology and Forensic Medicine, Al Kindy College of Medicine, University of Baghdad-00964, Iraq
Email: alaakasim@kmc.uobaghdad.edu.iq
Cell: 09647708840289

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