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Research Article

The Correlation of Expression of P16Ink4a and CD31 Immunohistochemical Stains in Invasive Squamous Cell Carcinoma of the Cervix and its Precursors

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ABSTRACT

Background: Squamous cell carcinoma of the cervix (SCC) and its precursors squamous intraepithelial lesions (SIL) remain a major global health burden. The tumor suppressor protein P16(Ink4a), a surrogate marker for high-risk human papilloma virus (HPV) infection, and CD31, an angiogenesis marker reflecting microvessel density (MVD), play critical roles in tumor progression.

Objective: This study aimed to assess the immunohistochemical (IHC) expression of P16(Ink4a) and CD31 in SIL and SCC and assess their correlation with disease severity.

Subjects and Methods: A cross-sectional study was conducted on 77 archived cervical biopsies (17 low-grade SIL [LSIL], 24 high-grade SIL [HSIL], and 36 SCC cases) from archival materials of two Baghdad hospitals (Medical City, The Teaching Hospital and Medical City of Al-Imamain AL-Kadhimein) (2018–2022). IHC staining for P16(Ink4a) (scored by intensity/distribution) and CD31 (for MVD quantification) was conducted. Statistical analysis used SPSS v26, with significance set at $p < 0.05$.

Results: P16(Ink4a) expression significantly increased from LSIL (35.3%) to HSIL (70.8%) and SCC (91.7%) ($p < 0.001$), with the highest scores in SCC (30.6%). MVD also rose with cervical lesion severity ($p < 0.001$), with means of 4.37 (LSIL), 13.11 (HSIL), and 52.81 (SCC). Poorly differentiated SCC showed the highest MVD (67.34). A strong positive correlation was found between P16(Ink4a) and MVD ($p < 0.001$).

Conclusions: Both P16(Ink4a) and CD31 (MVD) expression correlate strongly with cervical lesion severity, suggesting their potential as biomarkers for disease progression. Further studies are warranted to validate their prognostic utility in cervical cancer management.

Introduction

Cervical squamous cell carcinoma is the fourth most common type of cancer in women globally, accounting for 70–80% of all cases of cervical cancer [1].

In Iraq, there are299 new cases of cervical cancer were reported in 2022, according to the most recent Iraqi Cancer Registry Report [2].

The tumor suppressor protein P16(Ink4a) of the CDKN2A gene inhibits cyclin-dependent kinases 4 and 6. The down-regulating effect of P16(Ink4a) on cell proliferation is ineffective in cells infected by high-risk HPV [3] and previous studies conclude that P16 is directly related to the presence of HPV [4]. The degree of P16(Ink4a) expression and the viral genome's status have been proposed in a number of studies as potential diagnostic indicators for the advancement of cervical cancer [5-7].

The creation of new blood vessels from preexisting vascular structures, primarily capillaries and venules, under the influence of a malignant tumor is known as tumor angiogenesis [8] that often estimated using microvascular density (MVD), which is measured by labeling the vessels to be tallied, antibodies targeted any of the natural antigens expressed by endothelial cells, including FVIII, CD31, CD34, and CD105, are commonly used for this purpose [9]. The endothelial cell marker CD31 is a key marker of angiogenesis [10] and has been extensively used in previous cancer tissue studies, and this approach by using monoclonal antibodies against them [11].

The aim of this study is to examine the IHC expression of P16(Ink4a) and CD31 in the cervical squamous cell carcinoma and precursors squamous dysplastic lesion and to assess their correlation.

Subjects and Methods

In this cross-sectional study, a total of seventy seven cervical biopsies fixed in formalin and embedded in paraffin (total abdominal hysterectomy, cone and punch) of which 17 were diagnosed with LSIL and 24 with (HSIL, and 36 cases of SCC were selected from the archives of two teaching hospitals in Baghdad – Iraq (Medical City, The Teaching Hospital and Medical City of Al-Imamain AL-Kadhimein) for the period from January 2018 to January 2022.

An experienced pathologist made the initial histological diagnosis on the hematoxylin and eosin-stained slides. The demographics of the patients, the histological type of dysplasia, grade and stage according to the International Federation of Gynecology and Obstetrics (FIGO) of squamous cell carcinoma were all collected from their admission case sheets and pathology reports. Any sample lacking these clinicopathological characteristics was eliminated from this selection investigation.

For each case, two sections each four-micrometer thickness were immunohistochemically stained for P16(Ink4a) and CD31 after being placed on positively charged slide.

Anti-CD31 antibody, clone (EP3095), manufactured by Abcam, catalog number (ab134168), dilution (1:250) is intended for qualitative identification with light microscopy of CD31-positive cells in normal and neoplastic tissues, by using IHC test methods and monoclonal mouse antibodies.

Anti-P16(Ink4a) antibody, clone (2D9A12) manufactured by Abcam, catalog number (ab54210), dilution (1:200), is designed for use in laboratories to detect P16(Ink4a) positive cells by light microscopy in normal and neoplastic tissues using IHC test methods.

Slides were deparaffinized twice for 30 minutes using xylene and then rehydrated by gradually adding ethanol solution to Tris-buffered saline (TBS). The tissue sections were placed in 10 mM citrate buffer (pH 6.0) and heated in a hot air oven for 30 minutes at 95°C in order to perform epitope recovery. 10% H_2O_2 eliminated endogenous peroxidase activity. To prevent nonspecific binding sites, a protein blocker (Dako, Glostrup, Denmark) was employed. After one hour of room temperature incubation in a humidified chamber with a monoclonal antibody against human p16 and CD31, the slides were incubated in the EnVision Plus Dual Link System. The chromogen, 3,3′-diaminobenzidine (DAB), was employed for 30 minutes in the presence of hydrogen peroxide in HRP solution (Dako, Glostrup, Denmark). As a counterstain, Mayer's hematoxylin was used.

Interpretation and evaluation of IHC staining data:

CD31 positive response is shown by endothelial cells with brown cytoplasmic staining. Lymph node tissue serves as a control (as recommended by the manufacturer). By omitting the main antibody, a negative control was produced from a technical standpoint.

Weidner's approach would be used to compute intratumoral MVD for the scoring of CD31 immunohistochemical expression in situation of noninvasive and cancer tissue with fixed counting rules and magnification. After strict exclusion of non-tumoral areas three locations of interest will be chosen after low power (x10) scanning of tissue slices (The region with the densest vessel growth is known as the hot spot) (12). Only highly concentrated tumor cell clusters in healthy tissue (i.e., not sclerotic or necrotic) were included in this analysis. When assessing SILs, microvessel density was measured in the stroma beneath the basement membrane of the dysplastic epithelium (13). Individual blood microvessels were counted at a greater magnification when the area of interest (a vascular hot spot) was discovered. The number of microvessels was determined by magnifying an area of 0.74 mm^2 at power (x 20), which gives us the microvessel count (MVC). The MVD was calculated by averaging the fields measured at each of the three focal points and then dividing that number by the 0.74 mm area of the high-power field. Each microvessel was defined as a single, brownish-stained endothelial cell or a cluster of such cells that was clearly delineated from neighboring tumor cells and connective tissue components (12), and to reduce bias, the hotspots count performed by two independent observers who are blinded to clinical outcomes or diagnosis, then averaged.

Brown cytoplasmic and nuclear staining of P16 (ink4a) was deemed positive. Positive control was taken from an astrocytoma tissue (as recommended by the manufacturer). By omitting the primary antibody, a negative control was obtained technically.

According to Pakdel, F. et al. (3), the percentages of positive nuclear and cytoplasmic staining as well as staining intensity were taken into consideration when interpreting p16 immunostaining.

A scale of "0 (no staining), 1 (weak, focally positive), 2 (strong, focally positive or weak, diffusely positive), and 3 (strong, diffusely positive)" was used to calculate the intensity score.

The following scales were used to calculate the percentage of positive nuclear staining: 3 = 51-75%, 4 = 76-100%, 2 = 26-50%, and 1 = 1-25%, with final immunoreactive scores falling between 2 and 7.

To evaluate the correlation between the variables, the Spearman correlation coefficient was used, the mean ± SEM (standard error of the mean) was used to express continuous variables, while categorial variables were expressed as number and percentages. To test statistical relations between two categorial variables, Fisher exact tests or Chi-square were used. Unpaired t-test and ANOVA were used when relations between categorial and continuous variables were required. The Statistical Package for the Social Sciences (SPSS), version 26 (SPSS Inc., Chicago, IL, USA), was used for all calculations, and a p-value of less than 0.05 ($p<0.05$) is considered significant.

Results

The clinicopathological data of LSIL, HSIL and invasive SCC are demonstrated in Table (1). Considering the scoring of P16(ink4a) IHC expression, cases with the highest score [7] concentrated in those with SCC (30.6%) comparing to (12.5%) in those with HSIL and no case reported in LSIL, which is significant statistically (P=0.005). Table (3). The descriptive analysis also shows a significantly increase in P16(ink4a) expression from LSIL (35.3%) through HSIL (70.8%) to carcinoma (91.7%) (P < 0.001), table (2). However, no significant difference was reported in IHC expression of p16 (ink4a) in relation to different grades (p=0.849) and stages (p=0.570) of squamous cell carcinoma.

The mean MVD (measured by IHC expression of CD31) elevated considerably as cervical lesion severity increased (P<0.001). The mean MVD for LSIL, HSIL, and carcinoma (Figure 3) was 4.37±0.58, 13.11±0.99, and 52.81±2.39 correspondingly Table (4). The same table also illustrates a significant increase in MVD with advancement of stage of SCC (P < 0.001). The current series shows a significant increasing in MVD with decreasing degree of differentiation in cervical carcinoma cases (P < 0.001). The mean ± SEM of MVD in well differentiated, moderately differentiated, and poorly differentiated carcinomas were (32.16± 2.64), (49.07 ± 1.92) and (67.34± 2.63), respectively Table (4).

Correlation between P16 (INK4a) and CD31 IHC expression in SILs and SCC:

In the individuals under investigation, there was a significant positive correlation (P<0.001) between IHC expression of P16 (ink4a) and of MVD (identified by IHC expression of CD31). Table (5).

Table1: Clinicopathological parameters of cases with LSIL, HSIL and SCC

Parameters		Values	Percentage
Age: mean (range± SEM) years		LSIL 17	33.47±1.99 23-45
		HSIL24	42.92±1.95 25-60
		SCC 36	47.36±1.92 31-78
Histopathological diagnosis	LSIL	17	22.1%
	HSIL	24	31.2%
	SCC	36	46.7%
Stage of SCC	Stage I	10	33.3 %
	Stage II	12	40.0%
	Stage III	6	20.0%
	Stage IV	2	6.7%
Grade of SCC	Well differentiated	5	13.9 %
	Moderately differentiated	19	52.8%
	Poorly differentiated	12	33.3%

Table 2: Association of P16(ink4a) immunohistochemical expression with the clinical parameters of SCC and its precursor.

Parameters		Positive p16	Negative p16	Total No.	*P*-value
Histopathological diagnosis	LSIL	6(35.3%)	11(64.7%)	17	<0.001
	HSIL	17 (70.8%)	7 (29.2%)	24	
	SCC	33 (91.7%)	3 (8.3%)	36	
Stage of SCC	Stage I	9 (90%)	1 (10%)	10	0.849
	stage II	11 (91.7%)	1 (8.3%)	12	
	Stage III	6 (100.0%)	0 (0.0%)	6	
	Stage IV	2 (100.0%)	0 (0.0%)	2	
Grade of SCC	Well differentiated	4 (80.0%)	1 (20.0%)	5	0.570
	Moderately differentiated	18 (94.7%)	1 (5.3%)	19	
	Poorly differentiated	11 (91.7%)	1 (8.3%)	12	

Table 3: frequency of distribution of LSIL, HSIL and SCC of the cervix cases by immunohistochemical expression and scoring of immunostaining of P16(ink4a)

Expression and scoring of P16 ink4a	LSIL No (%)	HSIL No (%)	SCC No (%)
Negative	11 (64.7)	7 (29.2)	2 (5.6)
2	0 (0.0)	2 (8.3)	5 (13.9)
3	2 (11.8)	1 (4.2)	5 (13.9)
4	1 (5.9)	4 (16.7)	3 (8.3)
5	1 (5.9)	3 (12.5)	4 (11.1)
6	2 (11.8)	4 (16.7)	6 (16.7)
7	0 (0.0)	3 (12.5)	11(30.6)
Total	17	24	36
P value	0.005		

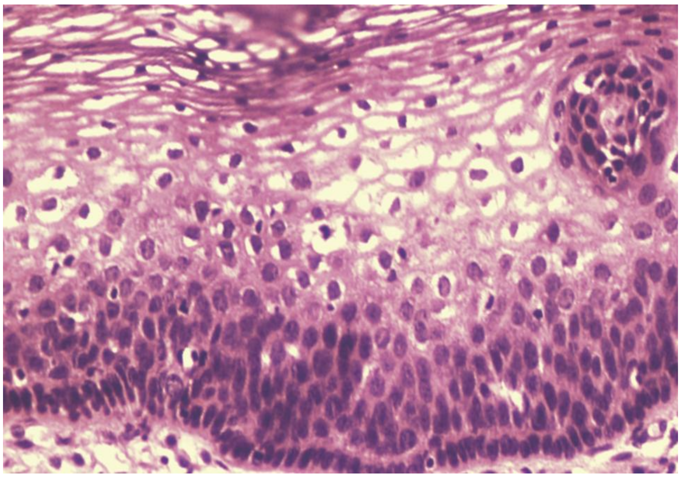

Figure 1: LSIL, shows dysplastic cells (hyperchromatic nuclei with high N/C ratio) restricted to the lower third of the epithelium, (H&E), (40x).

Table 4: Association of MVD detected by immunohistochemical expression of CD31 with the clinical parameters of SCC and its precursor.

Parameters		MVD mean+ SEM	*P*- value
Histopathological diagnosis	LSIL	4.37±0.58	<0.001
	HSIL	13.11±0.99	
	SCC	52.81±2.39	
Stage of SCC	Stage I	40.67± 2.83	<0.001
	stage II	57.31±2.37	
	Stage III	67.11±4.50	
	Stage IV	74.32±2.60	
Grade of SCC	Well differentiated	32.16±2.64	<0.001
	Moderately differentiated	49.07±1.92	
	Poorly differentiated	67.34±2.63	

Table 5: Correlation between IHC expression of p16 (ink4a), and CD31 in examined cases

Marker	P16(ink4a)	
	r	*P* -value
CD31 (MVD)	0.431	<0.001

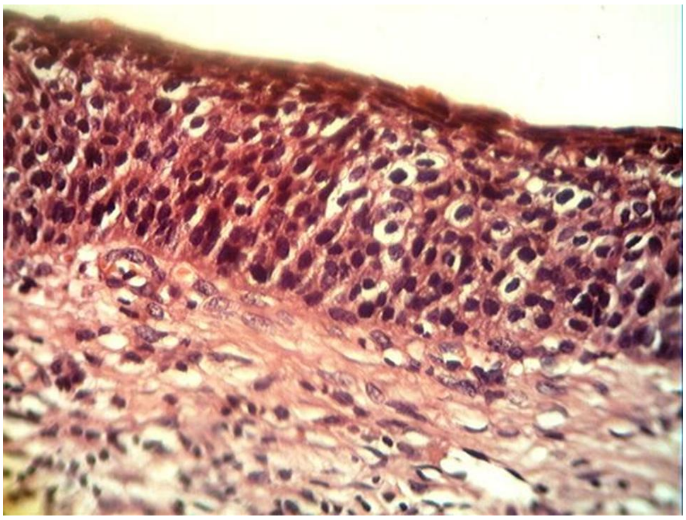

Figure 2: HSIL (CIN-III), shows dysplastic cells (hyperchromatic nuclei, high N/C ratio, pleomorphism, and mitosis) with lacking maturation throughout all layers, (H&E), (40x).

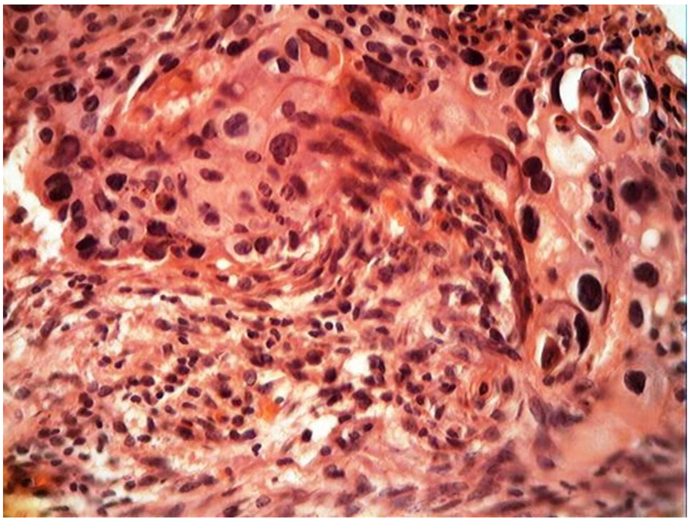

Figure 3: poorly differentiated squamous cell carcinoma displaying an irregular nest of malignant squamous cell nest (H&E), (40x).

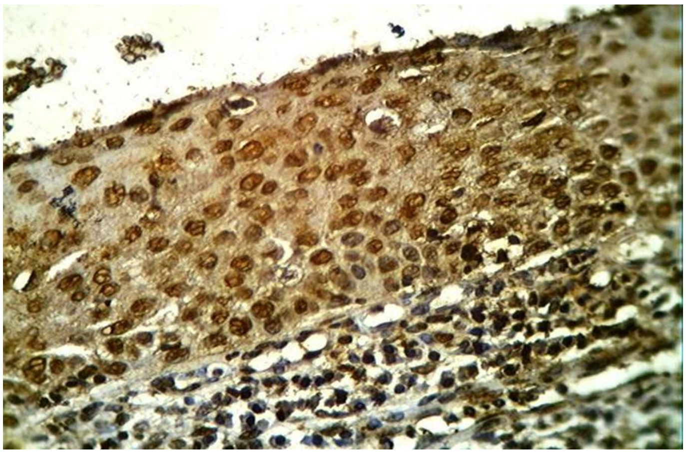

Figure4: Anti-p16(ink4a) monoclonal antibody immunohistochemical staining of the uterine cervix's HSIL demonstrates significant positive nuclear and cytoplasmic staining of the dysplastic cells with moderate intensity (2) and a high percentage (4) score (6) (arrow) (40x).

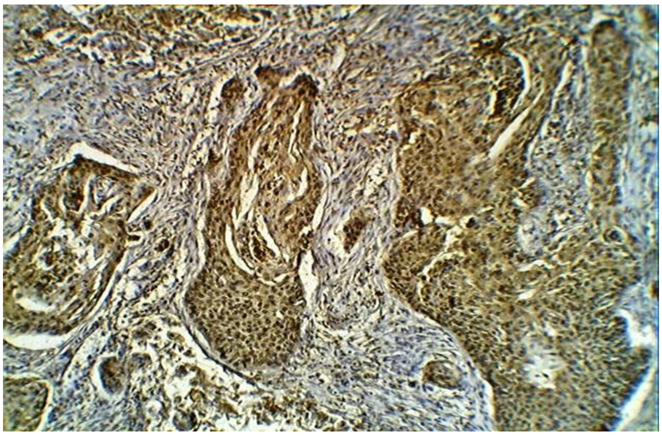

Figure 5: Anti-p16(ink4a) monoclonal antibody immunohistochemical staining of a moderately developed SCC of the uterine cervix demonstrates strong positive nuclear and cytoplasmic staining of the malignant cells

with high percentage (4) and great intensity (3), score (7) (arrow) (10x).

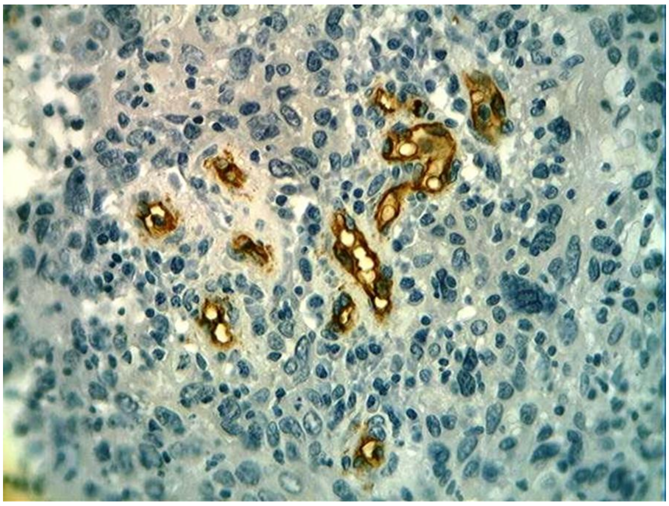

Figure 6: Anti-CD31 monoclonal antibody immunohistochemical staining of a poorly differentiated uterine cervical SCC shows a noticeable increase in intratumoral microvessel density (MVD) and positive cytoplasmic staining of microvascular endothelial cells (arrow). (40x)

Discussion

The p16 gene is a member of the INK4 family and consists of four members, P16(Ink4a), p15INK4b, p18INK4C and p19INK4D which all have similar biological characteristics i.e., tumor suppression and inhibition of cell growth. Mutations, methylation, and deletions of the p16 gene play important roles in tumorigenesis, progression, and metastasis (14–16).

When a patient contracts a high-risk HPV infection, the HPV oncoprotein E7 inactivates pRb, resulting in the loss of negative feedback and p16 overexpression (16).

The current study shows a significant elevation in P16 (ink4a) expression from LSIL through HSIL to carcinoma ($P < 0.001$). This agrees with Krishnappa et al. (2014) that found the intensity of p16-positive cells was increase with increasing grade of cervical abnormality (17), this suggests that the possible malignant transformation of cervical epithelial cells may be reflected in the overexpression of p16 (ink4a) (18). Increased overexpression of p16 (ink4a) could indicate more severe HPV inactivation of pRb, which would cause LSIL to proceed to higher grade (19).

Because HSIL and SCC express more P16(Ink4a) than lower grade lesions do, it can be used as a diagnostic marker to find high-risk lesions that could progress into invasive cancer.

Regarding cases of SCC, the current study shows no significant statistical difference in IHC expression of P16(Ink4a) among different stage and grade of the cases under investigation ($P>0.05$). These outcomes were determined by a meta-analysis by (Jiaying Lin et al.) (20).

An important marker for determining angiogenic activity in tumor is CD31, an endothelial cell marker that is involved in tumor angiogenesis and poor therapy response (10).

High CD31 levels were linked to increased tumor angiogenesis and poor treatment response, according to a study by Blank et al in 2015 (21).

Intratumoral microvessel density (iMVD) is a measure of angiogenesis which has been shown to be a prognostic indicator that correlates with increase risk of metastasis in various tumors.

CD31 IHC expression is among several techniques used for counting microvessel number (22). The current study shows that with increasing cervical lesion severity from LSIL to invasive carcinoma there was substantial rise in MVD (detected by IHC expression of CD31) ($P < 0.001$), a finding in tune with previous studies (11, 23).

Microvascular density proved to be an important predictor for worse prognosis as previous studies presented (24-26).

Stage of tumor is a very important parameter that determines the prognosis of any tumor (27), and this study demonstrates a substantial increase in MVD with increasing pathological FIGO stage in patients with SCC ($p<0.001$), a finding supported by previous study by Landt et al. (28).

Aijaz et al. (29) found that MVD distribution was higher in undifferentiated carcinoma compared to differentiated carcinoma, and this relationship was found to be statistically significant, a result come in tune with the current study.

Many Authors conclude that measuring MVD of tumors can reflect poor survival outcomes potentially identify it as a preferred marker of clinical significance (30).

VEGF overexpression is undoubtedly a component of the genetic dysregulation in the papilloma microenvironment, with patient-specific patterns. The viral oncoproteins E5, E6, and E7 are directly engaged in a number of the mechanisms underlying this overexpression. E6 and E7 interact with VEGF signaling in both direct and indirect ways. By activating the cellular ubiquitin ligase E6AP, E6 causes p53 to be degraded, whereas E7 activity activates the elongation factor 2 (E2F), increases the expression of cellular p16, and eventually promotes cell proliferation (31) and this may explain the positive correlation between IHC expression of P16(ink4a) and neoangiogenesis detected by IHC expression of CD31.

Conclusion

Angiogenic factors, such as VEGF (vascular endothelial growth factor), are upregulated in cervical cancer, promoting the formation of new blood vessels, which can be detected by many markers like CD31 offering a valuable tool for predicting tumor aggressiveness, metastasis risk, and survival outcomes in SCC.

P16(Ink4a) overexpression is a biomarker for HPV-associated cervical lesions and carcinoma and associated with advanced cervical cancer stages, where angiogenesis is more pronounced.

This correlation highlights the complex interplay between tumor suppressor pathways and angiogenesis in SCC progression.

Dual inhibition of HPV oncoprotein pathways (e.g., E6/E7) and angiogenic factors (e.g., VEGF) may disrupt carcinogenic synergy.

The retrospective and cross-sectional nature, relying on archival data from only two teaching hospitals in Baghdad, may limit the generalizability of the findings. The relatively small sample size may also affect the robustness and reproducibility of the results. Furthermore, the absence of molecular or HPV genotype restricts the ability to correlate histopathological findings with underlying etiological factors. Future studies with larger, a multicenter study with more diverse populations, adding normal cervical tissue as negative control, automated hot spot detection to objectively identify high-density vascular regions and the inclusion of molecular data (HPV genotyping) are recommended to fully elucidate the mechanisms linking P16(Ink4a) and angiogenesis in this context and improve statistical power.

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Conflict of Interest

Authors declare no conflict of interest.

Data availability

Data are available upon reasonable request.

Authors Contributions

MMA contributed to the conceptualization and methodology of the study. MA was responsible for data curation and funding acquisition. FK contributed to writing, reviewing, and editing of the manuscript. All authors read and approved the final version of the manuscript.

All authors meet the ICMJE criteria for authorship and agree to be accountable for all aspects of the work.

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