

HISTOLOGICAL AND MOLECULAR STUDY OF HOXB4 GENE IN CERVICAL CANCER AND BENIGN

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Abstract: Cervical cancer (CC) is one of the most common malignant tumors in women, with 604,000 new cases and 342,000 deaths in 2021 (1). Carcinoma of the cervix is the fourth most common health problem worldwide among women (2). Carcinoma cervix is caused by human papilloma virus (HPV), which is the most common sexually transmitted infection, has been shown to be related to UCC. This study was carried out in Laboratories of the College of Science/ Department of Biology and in a local lab during the period between December 2021 to August 2022. The study of PCR was used to detection *HOXB4* expression. We aimed to describe the histological changes of tumors malignant and benign and normal tissues of the cervical and determine the relationships between *HOXB4* expression with the histopathological variables involving, stage, grade, muscle and lymph nodes invasiveness and histological types. results shows the values of *HOXB4* expression and a significant difference (P-value<0.001) between patients with cervical cancer and control group, these results regarding the values means of *HOXB4* expression a significant difference (P-value=0.021) between patients with benign tumor and the control and the values means of *HOXB4* expression a significantly difference (P-value <0.001) between patients with cervical cancer and benign tumor.

Key word: cervical cancer, PCR, *HOXB4*.

1. Introduction

Cervical cancer (CC) is one of the most common malignant tumors in women, with 604,000 new cases and 342,000 deaths in 2021 (1). Carcinoma of the cervix is the fourth most common health problem worldwide among women (2). Carcinoma cervix is caused by human papilloma virus (HPV), which is the

most common sexually transmitted infection, has been shown to be related to UCC. There are more than 200 types of HPV. High-risk HPV types are associated with uterine cervical, vulvar, vaginal, penile, anal, and head and neck cancers (3). Magnetic resonance imaging (MRI) is the modality of choice for loco-regional staging of cervical cancer in the

primary diagnostic work-up starting with stage IB (4). MRI is the diagnostic imaging method of choice, not only for the pre-therapeutic local staging of cervical cancer, but also for the follow-up and evaluation of a treatments' success (5). Squamous cell carcinoma (SCC) and adenocarcinoma (AC) represent the major cervical cancer histotypes. Both histotypes are caused by infection with high-risk HPV (hrHPV) and are associated with deregulated microRNA expression (6). Uterine cervical fibroids are rare, accounting for about 5% of all myomas (7). Polyps of the uterine cervix are one of the most common benign hyperplastic lesions occurring in the female genital tract that usually arise from the endocervical canal, usually small in size (less than 4 cm) and malignant transformation in cervical polyps are considered to be very rare (8). *HOXB4* is a member of the HOX family of genes comprised of four clusters, namely HOX A, HOX B, HOX C, and HOXD (6). This gene is a member of the Antp homeobox family and encodes a nuclear protein with a homeobox DNA-binding domain. It is included in a cluster of homeobox B genes located on chromosome 17. The encoded protein functions as a sequence-specific transcription factor that is involved in development. Intracellular or ectopic expression of this protein expands hematopoietic stem and progenitor cells in vivo and in vitro, making it a potential candidate for therapeutic stem cell expansion (9). However, its biological function and exact mechanism in cervical cancer remain unknown, found that *HOXB4* was markedly downregulated in cervical cancer (10).

2. Material and Methods

2.1. Samples collection

This study included 45 samples of tissues were collected from cervix tumor patients, (15) samples were taken from patients diagnosed as CC with age ranged between 29 and 65 years, while the rest (20) were recorded as a benign tumor with age range between 40 and 60 years and 10 samples no cancer tissue ranged from 40 to 55 years were diagnosed as chronic

cervicitis and Nabothian cysts. All samples of patients were collected from Al-Zahraa and AL-Karama Teaching Hospitals in Wasit Province, and from Private Laboratory (BioTech) AL-Kut Province. This lab study was carried out in Laboratories of the College of Science/ Department of Biology and in a local lab during the period between December 2021 to August 2022.

2.2. Handling and Immediate Processing:

Upon retrieval, tissue samples were promptly immersed in 10% buffered formalin to ensure optimal fixation. Care was taken to mark the orientation of resected samples, especially in the case of larger specimens. Orientation markers, using sutures or dyes, helped in maintaining the histological context and facilitated accurate pathological interpretation later on.

2.3. Tissue Fixation

Ensuring the preservation of cellular and tissue architecture is pivotal for accurate histopathological assessment. The fixation process stabilizes tissue components, preventing post-sampling degradation.

2.4. Molecular studies of *HOXA11* gene in tumor endometrial

Total RNA were extracted from tissue samples by using RNA extraction protocol (GENEzol™ TriRNA Pure Kit, cat# GZX100/ D100) and done according to company instructions as following steps:

2.4.1. Deparaffinization

1. Five sections of 8 nm per 1.5- ml were transferred to Eppendorf tube and solubilized the paraffin twice with 1 mL of xylene for 5 mm at room temperature, then centrifuged at high-speed in a microcentrifuge for 10 mm and the pellet was saved after each time. The was removed su
2. pernatant carefully without loss of tissue fragments.
3. Samples were washed with 1 mL of absolute ethanol for 10 min and once with

ethanol 95%, then centrifuged in a microcentrifuge for 10 min and the pellet was saved after each time

4. Tissues were air-dry the with tubes open in a thermoblock at 37°C for approx 30 min.
5. Proteinase K was added to a final concentration of 6 mg/mL (43 µL of 20 mg/mL proteinase K every 100 µL of digestion solution).
6. Incubate overnight at 45°C with swirling.
7. Micro pestles were used to homogenized digested tissues.

2.4.2. RNA extraction

1. 400 µl of DR Buffer and 4 µl of β-mercaptoethanol was added to the tissue sample and then the sample was homogenized with a Tissue micro pestle, then tissue samples thoroughly.
2. Grinded tissue was transferred to a 1.5 ml microcentrifuge tube and 400 µl of DR Buffer and 4 µl of β-mercaptoethanol were added. Shear the tissue by passing the lysate through a 20-G needle syringe 10 times.
3. Samples were incubating lysate at room temperature for 5 minutes then centrifuged at 16,000 x g for 2 minutes. After that GD Column was placed in a 2 ml Collection Tube then supernatant was transferred to the the GD Column, and centrifuged at 16,000 x g for 1 minute.
4. Flow past the GD Column membrane following centrifugation, increase the centrifuge time until it passes completely. Save the flow-through in the 2 ml Collection Tube for RNA Purification.
5. GD Column was placed in a new 2 ml Collection Tube and stored at room temperature (15-25°C).
6. Elution Buffer (50 µl per sample) was heated to 60°C

2.4.3. RNA Purification

1. 0.8 volume of absolute ethanol was add to the flow-through in the 2 ml Collection

Tube and mix well by pipetting, then sample was transferred to the RB Column in a 2 ml Collection Tube and centrifuged at 16,000 x g for 1minute.

2. RB Column was placed the in a new 2 ml Collection Tube and 400 µl of RW1 Buffer into was added to the RB Column and centrifuge at 16,000 x g for 30 seconds. Discard the flow-through and place the RB Column back in the 2 ml Collection Tube. Add 600 µl of RPE Buffer (make sure ethanol was added) into the RB Column, centrifuged at 16,000 x g for 30 seconds.
3. Discard the flow-through and place the RB Column back in the 2 ml Collection Tube. Add 600 µl of RPE Buffer, and centrifuge at 14-16,000 x g for 30 seconds. Discard theflow-through and place the RB Column back in the 2 ml Collection Tube. Centrifuge at 16,000 x g for 3 minutes to dry the column matrix.
4. RB Column then placed in a clean 1.5 ml microcentrifuge tube (RNase-free). And 50 µl of RNase-free Water into the center of the column matrix and was left stand for at 3, and centrifuge at 16,000 x g for 1minute to elute the purified RNA.

2.4.4. Reverse Transcription

Reverse Transcription is carried out with the SuperScript First-Strand Synthesis System for RT-PCR (cDNA).The following procedure is based on Invitrogen's protocol.

1. RNA/Primer Mixture in each tube was prepare as shown in table (1):

Table (1): The components of RNA/Primer Mixture (cDNA) Prepare

Component	Volume (µL)
Total RNA	5 µg
random primer (0.1 µg/µl)	1 µl
2X reaction mix	10 µl
Enzyme mix	1 µl
RNAase Free water	3 µl
Total volume	20 µl

2. Reaction master mixture was prepared for each reaction as shown in table (2):

Table (2): Thermo cycler Program

Item	Step 1	Step 2	Step 3	Step 4
Temperature (C°)	25	42	85	4
Time	10 min	15 min	5 Min	∞

- The tubes were incubated at 25°C for 10 min as first step.
- Then tubes were incubated at 42°C for 15 min, heated at 85°C for 5 min, and then chilled on 4c.
- cDNA was stored at -20°C until use for real-time PCR.

2.4.5. Molecular detection for HOXB4 gene

2.4.5.1. Primers dilution

- The stock primers solutions (100 pmole/1µl) were prepared by adding NUECLASE FREE water in exact volumes as required by the manufacturer company.
- In order to achieve a final concentration of 10 pmoles/1µl, stock solutions for each primer were diluted with NUECLASE FREE water using a 1:9 ratio. Both forward and reverse primers were added in volumes of 1µl (10 pmole/1µl).

Table (3): Primers sequences of *HOXB4* gene

Primer	Sequence (5'-3')	Product size (bp)
HOXB4 F	GCAAAGAGCCCGTCGTCT	
HOXB4 R	GAAATTCCTTCTCCAGCT	
GAPDH F	TGCACCACCAACTGCTTAG	
GAPDH R	CAGGCAGGGATGATGTTC	

2.4.5.2. PCR working reaction

Components of PCR reaction and mixing amounts were shown in table (4).

Table (4): PCR components.

Component	Component of one sample
Sibergreen mastermix	12 µl
Forward primer	1 µM
Reverse primer	1 µM
cDNA template	5 ng/µl
Nuclease free water	1
Final volume	20

2.4.5.3. The PCR programs of *HOXB4* gene

A.PCR Programs of *HOXB4* gene

The PCR reaction was carried out as shown in Table (5) and Figure (1).

Table (5): PCR Programs of *HOXB4* gene

Steps	Temperature	Time	No. Of cycles
Initial denaturation	95 C°	10 min	1 cycle
Denaturation	95 C°	30 sec	40cycles
Annealing	58C°	40sec	
Extension	72 C°	30sec	
Final extension	25 C°	30sec	1 cycle
Holding	4 C°	Forever	-

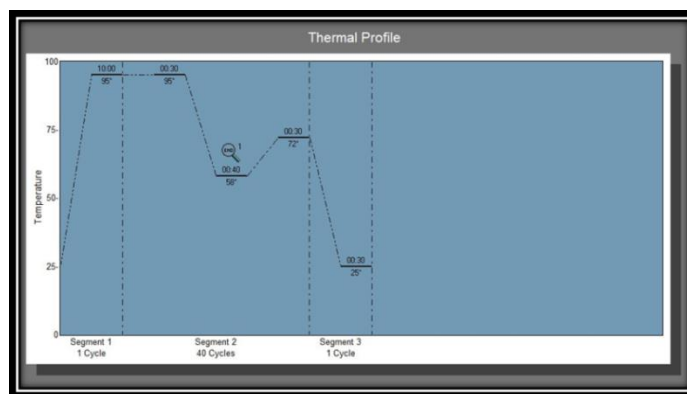


Figure (1): PCR Programs of HOXB4 gene

2.5: Statistical analysis

Data were entered and analyzed using the software program Statistical Package for Social Sciences (SPSS) version 26. All numerical variables were represented by means (a measure of central tendency) and standard deviation (a measure of dispersion) while categorical variables were presented by frequencies and percentages. Histograms and bar charts were used also to present variables. Independent samples t-test and one-way ANOVA test were used to assess the mean differences of numerical continuous variables accordingly. Fisher's Exact Test was used to assess the association between two categorical variables instead of chi-square test (more than 20% of the expected cells were less than 5). Considering a *P*-value equal to or less than 0.05 a significant.

3. Results and Discussion

3.1.1: Distribution of cervical patients according to aging group

This study include the age of the whole sample with cervical was represented, the mean age and standard deviation were 47.67 ± 8.17 years old and an involved 45 cervical patients, the minimum age was 29 years, while maximum age was 65 years.

This study shows the age group distribution among patients with cervical sample; age range between 29 and 65 years. The minimum age was 29 years, while maximum age was 65 years. The patients were a grouped into 4 age groups (1-4) with a (15) years interval. The first age group was (20-34) years, while the last one (65-80) years. Frequency of patients age group showed that 3 cases (6.7%) with age group 1(20-34), 25cases (55.6%) in age group 2 (35-49), 15 cases (33.3%) in age group 3 (50-64), 2 cases (4.4%) in age group 4 (65-80). These results also shown in figure (2) with age interval and table (6).

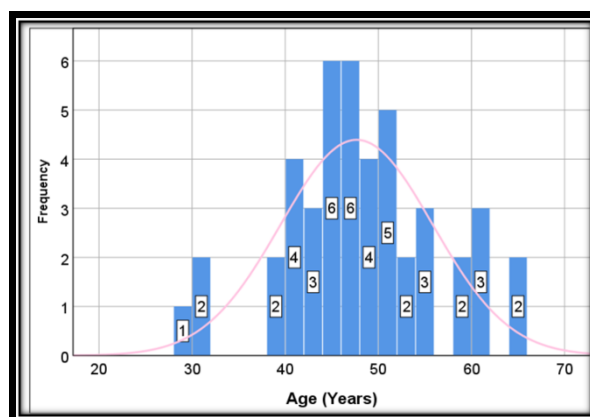


Figure (2): Histogram of the age distribution for 45 samples with cervical patients with age interval.

(Table6): Histogram of the age distribution for 45 samples with cervical patients with age interval.

Variable	Minimum Maximum	Mean	Standard Deviation
Age (Years)	29-65	47.67	8.17
	Age groups	Frequency	Percentage
	20-34	3	6.7%
	35-49	25	55.6%
	50-64	15	33.3%
	65-80	2	4.4%

This study showed that the highest incidence rate of patients with cervical in group 2 (35-49) years and formed 55.6% and group 3 (50-64) years and formed 33.3%. The age group 2 (35 - 49) years represented more than half (55.6%) of the sample. Followed by 33.3% for those group 3 (50 - 64) years old. Patients who were above 65 years and less than 80 represented only 4.4% of the total sample. Results of (11) showed that Among the study group, 90% (9 out of 10) women who had abnormal cervical biopsy were in the age-group of 35 years and above, about 70% of the women with abnormal histopathological findings had a prolonged duration of marriage of at least (20–30) years which came out to be statistically significant with a p-value of 0.033. This result in concordant with result (12) showed that the characteristics of cervical cancer patients based on age in 82 diagnosed cases and showed that the majority of cervical cancer patients were more than 35 years old. Also, (13) showed that the median age of all patients was 47.0 years (range 19 - 80). Moreover, (14) found that patients with normal cervix in age group (<30), (30-39), (40-49), (50-59), (60-69) and (≥ 70) years were diagnosed (0,10,28,17,13,1) of 69 cases respectively.

3.1.2: Distribution of cervical patients according to histopathology diagnosis among 45 patients.

In this study, it was illustrates distribution for 45 samples with cervical patients according to the diagnosis. 15(33.3%) patients with cervical tumor cancer (malignant tumors), 20(44.4%) patients with cervical benign tumors and 10 (22.2%) patients with no pathological changes in their cervical and those were complaining of control cervical (chronic cervicitis and nabothian cysts).

In this study, it was found that mean age and standard deviation with cervical patients samples (46.07 ± 11.30) patients of them were cervical cancer tumors, (49.45 ± 6.46) patients with cervical benign tumors and (46.50 ± 5.16) patients with cervix control Table (7). Distribution of cervical patients according to histopathology diagnosis among 45 patients.

(Table7): Distribution of cervical patients according to histopathology diagnosis among 45 patients.

Histopathology Diagnosis	Frequency	Percentage	Mean age	S.D	P-value
Cervical cancer	15	33.3%	46.07	11.30	0.430
Benign cervical tumor	20	44.4%	49.45	6.46	
Normal cervix (chronic cervicitis and nabothian cysts)	10	22.2%	46.50	5.16	
Total	45	100%			

These results showed no significant difference (P-value=0.430) between the three diagnosed groups of patients regarding age. Slightly similar mean ages were found between age groups ranging from 46.07 ± 11.30 for those who were diagnosed with cervical cancer and 49.45 ± 6.46 for patients with a benign tumor and with control group conditions were with a mean age of 46.50 ± 5.16 . Results of (15) found that 106 cases were also categorized according to histopathology, with staining by H&E stain

and implanted in paraffin wax, 75 (71%) were reported to be Invasive cervical carcinoma and 31 (29%) were reported to be cervical intraepithelial neoplasms. However, (16) showed that among the 500 conventional Pap smears screened, 422 (84.4%) cases were NILM of which 212 (42.4%) were normal, 206 (41.2%) were inflammatory and 4 (0.8%) were atrophic. Also, (17) showed that the malignant cases reported were in between (41-60) years of age group, the most common presenting complaint was menorrhagia, out of which 41% cases were diagnosed as leiomyoma, 15% cases were of adenomyosis and 8% cases consisting of both leiomyoma with adenomyosis and showed histopathological findings of cervix non-neoplastic and benign 129 (77.71%), pre-malignant 04 (02.40%) and malignant 02 (01.20%).

Malignant

3.1.3: Distribution of malignant cervical patients according to histopathological type

Five histological types were determined in the cervical cancer patients. The most frequent types were Squamotristational carcinoma and Squamous cell carcinoma patients with the same percentage of 4 (26.7%). Non-keratinized Squamotristational cell carcinoma and Endocervical adenocarcinoma, both of them represented about 3 (20%) patients of the total sample with cervical cancer, 1 (6.6%) patients with keratinized squamous cell carcinoma (Table 8). Distribution of malignant endometrial patients according to histopathological type.

(Table 8): Distribution of malignant endometrial patients according to histopathological type

Histotype	Frequency	Percentage
Squamotristational carcinoma	4	26.7%
Non-keratinized squamotristational cell carcinoma	3	20%
Squamous cell carcinoma	4	26.7%
Endocervical adenocarcinoma	3	20%
keratinized squamous cell carcinoma	1	6.6%

In the results are that related to pathological type, five histotypes of cervical cancer were identified in the study sample. The most frequent types were Squamotristational carcinoma and Squamous cell carcinoma with the same percentage of 26.7%. Non-keratinized squamotristational cell carcinoma and Endocervical adenocarcinoma, both of them represented about 20% of the total sample with cervical cancer. Only one out of 15 (6.6%) was diagnosed with keratinized squamous cell carcinoma. This result is concordant with result (18) showed that the characteristic based on the type of histopathological findings, squamous cell carcinoma is the most commonly found histopathological characteristic with 34 people, (85%) followed by adenocarcinoma with 4 people (10%), and adenosquamous carcinoma with 2 people (5%). Also, (19) showed that related to the characteristics of cervical cancer patients based on histopathological findings in 82 cervical cancer cases which showed that squamous cell carcinoma was more commonly found than adenocarcinoma with a total of 52 cases (63.4%). In addition, (15) explain that Out of 75 cases, 71 (94.67%) cases were of squamous cell carcinoma type and 4 (5.33%) cases were of adenocarcinoma type. Among the 71 squamous cell carcinoma cases (SCC), 60 (84.51%) cases were diagnosed as Large Cell Non- Keratinizing SCC, 7 (9.86%) cases were diagnosed as Large Cell Keratinizing SCC, and 4 (5.63%) cases as Small Cell Non-Keratinizing SCC. In addition, (20) reported that histopathology confirmed 299 (60.3%) squamous cell carcinomas, 169 (34.1%) adenocarcinomas, and 28 (5.6%) adenosquamous cell carcinomas. However, (13) showed that The majority of histological subtype was squamous cell carcinoma (2,936, 89.0%), and adenocarcinoma and adenosquamous carcinoma were found in 245 (7.4%) and 117 (3.5%) patients, respectively.

3.1.4: Association between age group and histotype of malignant endometrial tumor patients.

This study involved malignant cervical tumor patients; age range between 29 and 65 years. The minimum age was 29 years, while maximum age was 65 years. Malignant cervical tumor patients were grouped into 4 age groups (1-4) with a (15) years interval. The first age group was (20-34) years, while the last (65-80) years. Estimation of malignant cervical age group showed that 0 cases (0.0%) with age group 1(20-34) and age group 2 (35-49), 3 cases (50.0%) with age group 3(50-64) and 1 case (50.0%) with age group 4 (65-80) of them were Squamotras-tional carcinoma, 3 cases (33.3%) with age group 1(20-34), 1 case (25.0%) and 1 case (16.7%) with age group 2(35-49) and group 4 (50-64) of them were Non-keratinized Squamotras-tional cell carcinoma, 1 case (33.3%) with age group 1(20-34), 2 cases (50.0%) with age group 2 (35-49) and 1 case (50.0%) with age group 4(65-80) of them were Squamous cell carcinoma. 1 case (25.0%) with age group 2 (35-49) and 2 cases (33.3%) with age group 3(50-64) of them were Endocervical adenocarcinoma. 1 case (33.3%) in age group 1(20-34) with keratinized squamous cell carcinoma.(Table 9). Association between age group and histotype of malignant endometrial tumor patient.

(Table 9): Association between age group and histotype of malignant cervical tumor patients.

Age category (years)	Histotype					P-value
	Squamotras-tional carcinoma	Non-keratinized Squamotras-tional cell carcinoma	Squamous cell carcinoma	Endocervical adenocarcinoma	keratinized squamous cell carcinoma	
20-34	0(0%)	1(33.3%)	1(33.3%)	0(0%)	1(33.3%)	0.400
35-49	0(0%)	1(25%)	2(50%)	1(25%)	0(0%)	
50-64	3(50%)	1(16.7%)	0(0%)	2(33.3%)	0(0%)	
65-80	1(50%)	0(0%)	1(50%)	0(0%)	0(0%)	

The results are that related to Age in table (9) was not significantly associated with the histotype of cervical cancer (P-value=4.00) histotypes were distributed among age groups slightly in the same manner. The three patients aged below 34 years who had cervical cancer were distributed to three histotypes including 1(33.3%) (Non-keratinized Squamotras-tional cell carcinoma, Squamous cell carcinoma and keratinized squamous cell carcinoma). The four patients aged below 49 years who had cervical cancer were distributed to three histotypes including 1(25.0%) (Non-keratinized Squamotras-tional cell carcinoma and Endocervical adenocarcinoma) and 2(50.0%) Squamous cell carcinoma, adenocarcinoma. The six patients aged below 64 years who had cervical cancer were distributed to three histotypes including 3(50.0%) Squamotras-tional carcinoma, 1(16.7%) Non-keratinized Squamotras-tional cell carcinoma and 2(33.3%) Endocervical adenocarcinoma. The two patients aged below 80 years who had cervical cancer were 1(50.0%) Squamotras-tional carcinoma and 1(50.0%) Squamous cell carcinoma. Also, (21) explain the sample based on age, from 40 samples, the highest cases were in the 40-49 year age group as many as 18 people (45%), followed by the 50-59 year age group (32.5%), the <40 year age group. (17.5%), and at least 2 people (5%).

3.1.5: Frequency distribution of the cervical cancer patients according to grading.

In this study involved 15 cervical cancer patients is divided into three grades, grade I was appeared in 7 (46.7%) of patients, grade II found in 3(20.0%) of patients while grade III was presented in 5 (33.3%) of patients (Table 10): Frequency distribution of the cervical cancer patients according to grading.

(Table10): Frequency distribution of the cervical cancer patients according to grading.

Grade	Frequency	Percentage
G1	7	46.7%
G2	3	20.0%
G3	5	33.3%
Total	15	100%

The results grading of cervical cancer among patients in this study was shown the proportion of patients in grade I is higher if compared with grade II and grade III, revealed that the majority proportion of patients in grade I 7(46.7%) followed by 5(33.3%) for grade III and only 3(20.0)% were belonging to grade II. This result in discordant with result (13) showed that patients were categorized according to their grades, in SCC, 3(4.2%) cases were of the well-differentiated type, 63(88.7%) were moderately differentiated and 05(7.0%) cases were poorly differentiated and all four cases of adenocarcinoma were moderately differentiated. Moreover, (22) found that histological grading was grade III (poorly or non-differentiated carcinoma), grade II (moderately differentiated), and grade I (well-differentiated) in 15 (45.5%), 4 (12.1%), and 14 (42.4%) patients, respectively.

3.1.6: Frequency distribution of the cervical cancer patients according to staging.

In this study, it was found that cervical cancer patients are divided into four stages, stage I was appeared in 11 (73.3%) of patients, stage II found in 4 (26.7%) of patients while stage III and stage IV found in 0 (0%) of patients Table (11): Frequency distribution of the cervical cancer according to staging.

Table (11): Frequency distribution of the cervical cancer patients according to staging.

Stage	Frequency	Percentage
I	11	73.3%
II	4	26.7%
III	0	0%
IV	0	0%

The results staging of cervical cancer among patients in this study was regarding the staging proportion of patients cancer in stage I is higher if compared with stage II, stage III and stage IV. The majority portion 11(73.3%) were from stage I followed by stage II 4(26.7%) and no one presented with stage III and IV. Results of (13) showed that the specific FIGO stage of all the patients were listed as follows: IB1 (1,165, 35.3%) cases, IB2 (308, 9.3%) cases, IIA1 (1,252, 38.0%) cases and IIA2 (573, 17.4%). However, (23) explained that Nearly one fifth of patients (n = 2,171, 17.4%) were ≥ 65 years, more women ages ≥ 65 years (71%) presented with late-stage disease than younger women (48% in patients ages < 65). Late-stage 5-year relative survival was lower for women ≥ 65 years (23.2%–36.8%) compared with patients < 65 (41.5%–51.5%), characteristics associated with late-stage cervical cancer in women ≥ 65 years included older age. Also, (24) found that the majority portion 84 (64.6%) were from stage III followed by stage I–II 36 (27.7%) and 10 (7.7%) with stage IV.

3.6: Frequency distribution of stromal invasiveness among 15 patients diagnosed with malignant cervical cancer

Cervix cancer is either invasive or non-invasive stromal. In this study, it was found that 4(26.67%) of patients suffering from stromal invasiveness cervical cancer, and other 11 (73.33%) of them presented with non-invasive Figure (3): Frequency distribution of stromal invasiveness among 15 patients diagnosed with malignant cervical cancer.

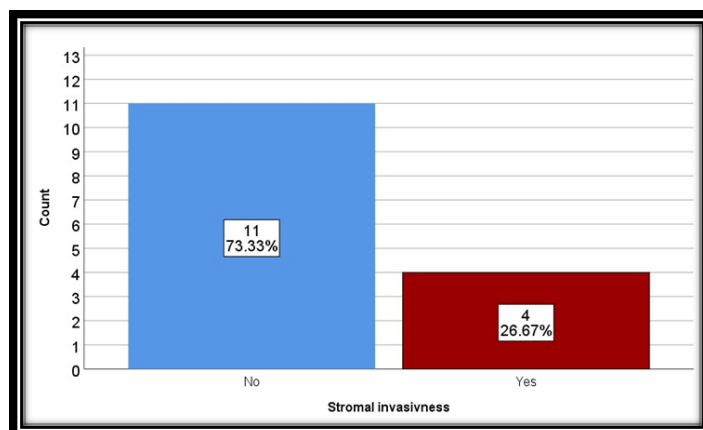


Figure (3): Frequency distribution of stromal invasiveness among 15 patients diagnosed

The results of this figure (3.4) showed that proportion absent stromal invasion in cervical cancer patients is higher compared with present invasion stromal. Stromal invasiveness wasn't found in 11 patients (73.3%) and only 4 out of 15 (26.6%) cancer patients reported stromal invasiveness patients who were diagnosed with cervical cancer in this study. Results of (25) found Invasion depths of $<1/2$ and $\geq 1/2$ of the stroma were observed in 168 (59.2%) and 116 (40.8%) cases, respectively. However, (13) 241 showed approximately 60.6% (1998/3298) of the cases presented with outer middle third to full-thickness stromal invasion, 33.5% (1106/3298) of the patients were full-thickness stromal invasion, and only 5.9% (194/3298) patients had outer full-thickness invasion. Also, (26) found the median depth of stromal invasion was 20.5(0–100%) in the cancer-invisible group and 63.5% (0–100%) in the cancer-visible group ($p < 0.001$).

3.1.7: Frequency distribution of the lymph node involvement among 15 patients diagnosed with malignant cervical cancer.

Cervix cancer is either invasive or non-invasive lymph node. In this study, it was found that 15 (100%) of patients suffering from non-invasive lymph node invasiveness cervical cancer, and other 0 (0.0%) of them presented with invasive lymph node Table (12): Frequency distribution of the lymph node involvement among 15 patients diagnosed with malignant endometrial cancer.

Table (12): Frequency distribution of the lymph node involvement among 15 patients diagnosed with malignant endometrial cancer

Variable	Yes Number (%)	No Number (%)
Lymph node involvement	0(0%)	15(100%)

The results of table (12) showed that the proportion absent lymph node invasion in cervical cancer patients is higher compared with lymph node invasion if all cases of malignant cervical tumors (100%) had no lymph node involvement. This may be patients were diagnosis with early-stage from cervical cancer. This result in concordant with result (27) who found para-aortic lymph node metastasis was detected in 45 of 204 patients with early cervical cancer, with a positive rate of 22.06%, aged from 26 to 66 years and non-metastatic was 159 patients. In addition, (20) reported that the incidence of lymph node metastasis was 4.6% (23/496) also more common in patients with deep stromal invasion ($P < 0.001$) and 44 (86.3%) patients without lymph node metastasis. However, (28) reported that among three hundred twenty-one these patients, 280 did not present lymph node invasion (87.2%), 13 presented isolated tumor cells (4%), 11 presented had lymph nodal micrometastases (3.4%) and 17 had lymph nodal macrometastases (5.3%).

Benign

3.1.8: Distribution of benign cervical patients according to histopathological type

In this study, it was illustrates distribution for 20 patients who were diagnosed with a benign tumor in the cervix. Two histological types were determined in the benign patients. 6 (30.0%) patients of them were cervical leiomyoma leiomyoma, 14 (70.0%) patients with endocervical polyp (Table13): Distribution benign cervical patients according to histopathological type.

Table (13): Distribution benign cervical patients according to histopathological type

Histotype	Frequency	Percent
Cervical leiomyoma	6	30%
Endocervical polyp	14	70%
Total	20	100%

This study showed that among the 20 patients who were diagnosed with a benign tumor in the cervical, the majority and highest rate been in 14(70%) were cervical leiomyoma while the remaining 6(30.0%) were endocervical polyp and the results showed that endocervical polyp is most common. Result of (29) found that histopathological examination in cervix, the commonest pathology was found to be chronic cervicitis 41(82%) cases followed by papillary endocervicitis 29 (58%) cases, leiomyoma 25 (50%) and Nabothian cyst 12 (24.5%) cases. Also, (30) showed that majority of the patients 54 cases (24.32%) presented with menorrhagia followed by uterovaginal prolapse with 53 cases (23.87%), cervical dysplasia 2 cases (00.90 (% and less common presenting features were cervical polyp in 1 case (0.45%).

3.1. 9: Association between age group and histotype of the benign cervical tumor.

This study involved benign patients; age range between 35 and 64 years. The minimum age was 35 years, while maximum age was 64 years. The benign patients were a grouped into 2 age groups (1-2) with a (15) years interval. The first age group was (35-49) years, while the last (50-64) years. Estimation of benign age group showed that 6(46.2%) cases with cervical leiomyoma while 7(53.8%)cases with endocervical polyp with age group 1(35-49), 0(0%) cases with cervical leiomyoma while 7(100.0%) cases with endocervical polyp with age group 2(50-64) Table(14): Association between age group and histotype of the benign endometrial tumor.

Table (14): Association between age group and histotype of the benign cervical tumor.

Age category (years)	Histotype		P-value
	Cervical Leiomyoma	Endocervical polyp	
35-49	6(46.2%)	7(53.8%)	0.051
50-64	0(0%)	7(100%)	

This study showed that, there was not significantly association between the age groups of patients with benign tumors and their histotype (P-value=0.051). Patients in the age group (35-49) years were diagnosed 6(46.2%) with cervical leiomyoma and 7(53.8%) with an endocervical polyp while all patients above 50 7(100%) were diagnosed with an endocervical polyp. These results agreed with the results (31) showed that among 214 patients cervical leiomyomas, the weighted average age was 39.4 years-old. Moreover, (35) found that patients with cervical polyp in age group (<30), (30-39), (40-49), (50-59), (60-69) and (≥ 70) years were diagnosed (3,5,12,2,3,1) of 26 cases respectively.

3.2: Histological Structure

3.2.: Microscopical Structure of Cervix Tissues

3.2.1.1: Microscopical Structure of Cervix control

The histological examination of sections of cervix tissues with H&E stain was appeared as the cervix tissues consists of two layers: the epithelium (exocervix and endocervix) and the stroma, exocervix is lined by stratified squamous epithelium and the endocervix is lined by columnar mucinous epithelium, the squamocolumnar junction (SCJ) is the region that marks the boundary and transformation zone is the hormonally responsive zone of metaplasia between the ectocervix and endocervix and considered as precise histologic transition between squamous and glandular epithelium these zone of the cervix predominantly involved dense lymphoplasmacytic inflammation with or without lymphoid follicle formation called Nabothian cysts (also named mucinous retention cysts or epithelial cysts), these cysts filled with mucus, but they may also contain proteinaceous material, neutrophils, or neutrophil debris and these cysts usually seem superficially and are easily diagnosed through colposcopy examination(Figur 4). This characteristic was identical for finding of (Barrigón et al., 2019) suggests that these cysts are at the squamocolumnar junction (SCJ) of the uterine cervix, which is the targeted anatomical area of brush sampling at the time of cervical screening cytology. Also, (32) explain that the squamocolumnar junction (SCJ) is the region that marks the boundary between the ectocervix and endocervix also considered dynamic in location, which changes throughout the reproductive years and explained cervix tissues consists of two layers: the epithelium (ecocervix and endocervix) and the stroma, ecocervix is lined by stratified squamous epithelium and the endocervix is lined by columnar mucinous epithelium.

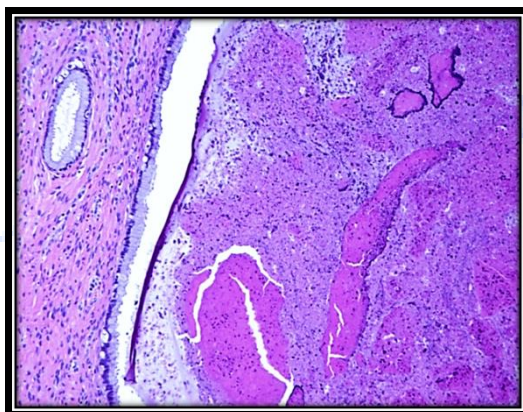


Figure: (4): Nabothian cysts with H&E stains (10X).

3.2.1.2: Microscopical Structure of Cervix Malignant

The histological examination of sections of cervix malignant tissues with H&E stain was appeared as cervical cancer is originate in the transformation zone, which surrounds the external os; the transformation zone is an area of squamous metaplasia between the original and current squamocolumnar junction, cervical cancer is usually squamous cell carcinoma; adenocarcinoma is less common. The cause of most cervical cancers is human papillomavirus (HPV) infection. Cervical cancer is classified into many histological subtypes:

A- Cervical squamous cell carcinoma (CESC)

The histological examination of sections of in cervical squamous cell carcinoma tissue with H&E stain was appeared the tumor cells infiltrating as irregular anastomosing nests or single cells within desmoplastic or inflammatory stroma, stromal loosening, desmoplasia or increased epithelial cell cytoplasmic eosinophilia in tumors with superficial stromal invasion lymphovascular invasion may be

present. revealed infiltrative growth of moderately/highly atypical squamous epithelial cells forming mostly solid, nest and trabecular patterns growth with variable necrotic areas that extends more than 2cm, associated with moderate mixed inflammatory cells (mostly lymphocytes) (Figur5). This characteristic was identical for finding of (33) suggests that Microscopically, it was a neoplastic tissue with a solid architecture consisting of polymorphous tumor cells containing giant partly lobulated, multiple nuclei, and prominent eosinophilic nucleoli.

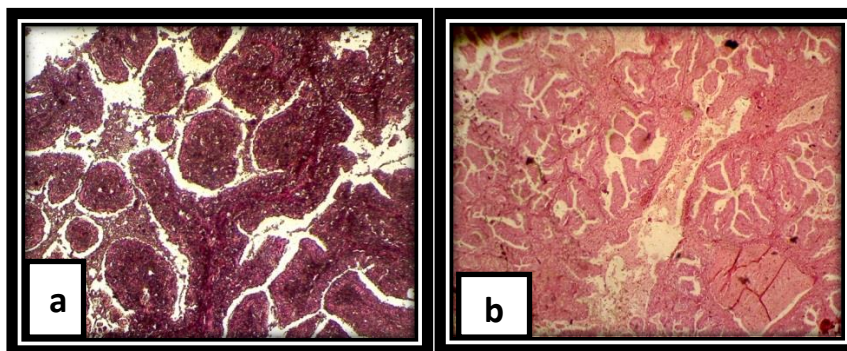


Figure (5): Cervical squamous cell carcinoma tissue with H&E stain a.(10X), b.(4X).

B- Endocervical adenocarcinoma (ADC)

The histological examination of sections of in cervical adenocarcinoma tissue with H&E stain was appeared as stromal infiltration in the form of marked glandular confluence with cribriform or microacinar architecture, irregularly shaped, angulated or fragmented glands with an adjacent desmoplastic stromal reaction, tumor cell clusters or individual cells lymphovascular space invasion. Increased number of glands with loss of a lobular arrangement and glandular density exceeding that of the normal cervix and glands are often close to thick walled vessels. As well as histological features reveled mucin depleted epithelium (comprise <50% of the tumor volume) in turn, most of population has columnar, no mucinous indistinct cytoplasm, cells have columnar shape; nuclei are elongated, enlarged and hyperchromatic with coarse chromatin with loss of polarity and nuclear overlapping associated with several mitotic activity, mitotic figures are atypical (Figur 6). This characteristic was identical for finding of (34) found that characteristically, the tumor cells of usual-type endocervical adenocarcinomas are columnar, with pseudostratified elongated and hyperchromatic nuclei. The cytoplasm is usually mucin-depleted, and the presence of apical mitotic figures and basal apoptotic bodies is virtually pathognomonic (although not seen in every case), can mimic adenosquamous carcinomas or endometrioid adenocarcinomas with squamous differentiation, the stroma is often, but not always desmoplastic, and sometimes there is an accompanying inflammatory infiltrate, necrosis, or pools of mucin.

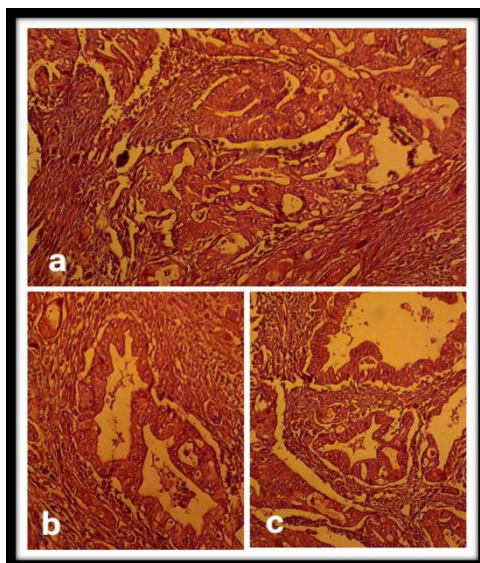


Figure: (6): Cervical adenocarcinoma tissue with H&E stains (10X).

C- keratinized squamous cell carcinoma

The histological examination of sections of keratinized squamous cell carcinoma cervix tissue with H&E stain was appeared as irregular anastomosing nests of large atypical hyperchromatic cells in desmoplastic inflamed stroma with increase cytoplasmic eosinophilic with keratohyalin granules and occational of keratin pearl, keratin pearls, abundant keratohyaline granules also intercellular bridges large, hyperchromatic nuclei with coarse chromatin and inconspicuous nucleoli (Figur7). This characteristic was identical for finding of (36) found that Keratinizing SCC is characterized by well-differentiated squamous cells infiltrating as nests, cords, and sheets of cells, the cells have abundant cytoplasm, large pleomorphic nuclei, and inconspicuous nucleoli. Keratin pearls and intercellular bridges are evident, the presence of even one keratin pearl has been considered sufficient for diagnosis, keratohyaline granules and individual cell keratinization are also seen. Also, this result similar to finding of (37).

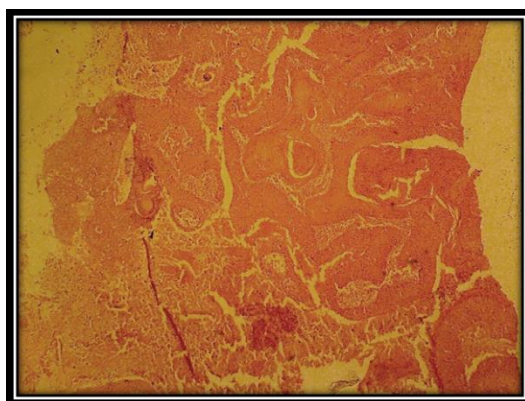


Figure: (7): keratinized squamous cell carcinoma tissue with H&E stains (10X).

D- Non-keratinized squamotrastional cell carcinoma

The histological examination of sections of Non-keratinized squamous cell carcinoma cervix tissue with H&E stain was showed invasive growth of squamous epithelial cells in the cervix large cells of similar size and shape with indistinct cell borders, infiltrating as nests and sheets and forming mostly solid, papillary, trabeculate, adenoid cystic like and pseudoglandlar pattern without keratinization. Also pleomorphic cells. Nuclei with prominent and mitotic figures are common. The cytoplasm is

moderate in amount and eosinophilic to amphophilic but keratin pearl formation should be absent (Figur8). This result was identical for finding of (36) similar to finding of (37).

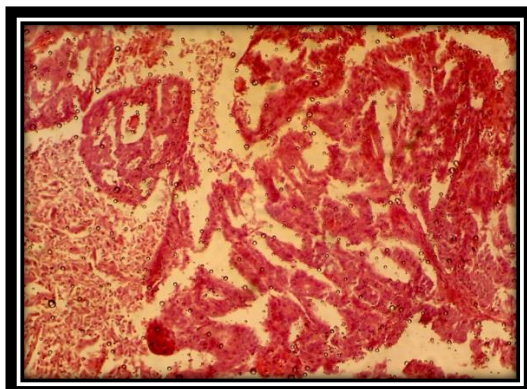


Figure: (8): Non-keratinized squamotrastional cell carcinoma tissue with H&E stains (4X).

E-Squamotrastional carcinoma of cervix (Papillary Squamotransitional Cell Carcinoma Cervix)

The histological examination of sections of Squamotrastional carcinoma of cervix tissue with H&E stain was showed invasive growth of squamous-trastional with fiberovascular core papillary configuration, associated with foci of well differentiated atypical squamous epithelium (Figur9). This result was identical for result of (38) found that mixed cellular components of transistional and squamous cells are seen with presence of papillary architecture at places. Moreover, (39) explain that tissue with groups of glands exhibiting moderate structural and cellular atypia and diffuse infiltration of tumor cells consisting of micropapillae, nests, and strands, while the cells had eosinophilic and light cytoplasm, large hyperchromatic nuclei with euchromatin, and oval and polygonal shapes.

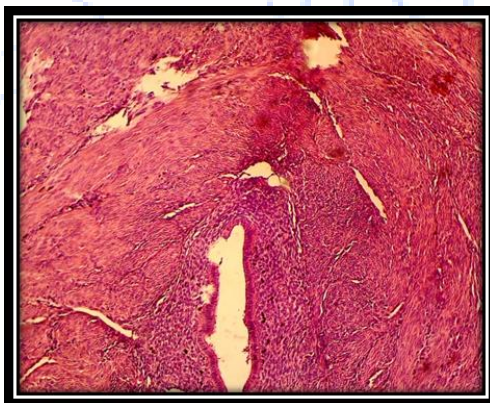


Figure: (9): Squamotrastional carcinoma of cervix tissue with H&E stains (10X).

3.2.1.3: Microscopical Structure of Cervix Benign

A-Endometrial polyp

The histological examination of sections of cervix benign tissues with H&E stain was appeared as cellular cervical leiomyoma intersecting bundles of bland looking smooth muscle cells having middle typical nuclear changes. There was degenerated tissue, the surface epithelium lined by simple columnar epithelium (Figur10). This result was identical for result of (40) found that histopathological characteristic of intramural leiomyoma benign smooth muscles arranged in an interlacing pattern with nosigns of nuclear atypia and large areas of hyalinization, nodules of varying sizes of uniform smooth muscles arranged in interlacing and whirling fascicles with few prominent blood vessels .

On the other hand, the histological examination of sections of cervical polyp tissue with H&E stain was appeared as polypoid of tissue with papillary projections lined by simple columnar endocervical type epithelium with underlying loose stroma often with mixed chronic inflammation in between dilated benign looking endocervical glands (Figur 11). This result was identical for result of (41) showed that stromal overgrowth and reactive epithelial hyperplasia associated with repeated episodes of inflammation, an abnormal local response to increased estrogen levels, and the local congestion of cervical stromal blood vessels. Also, (42) described as the presence of expelled fragments of the decidua at the cervix, both the gland and stroma exhibit decidual changes. Additionally, concomitant characteristics that are exhibited include endometrial polyps, erosion, inflammation, necrosis, hemorrhage, and thrombi.

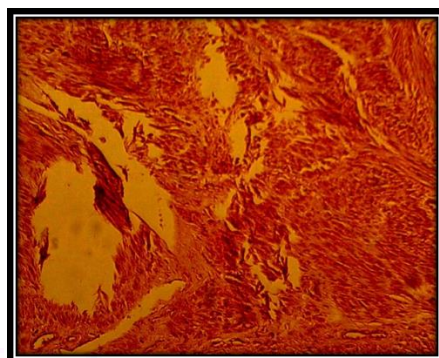


Figure: (10): Cervical leiomyoma tissue with H&E stains (10X).

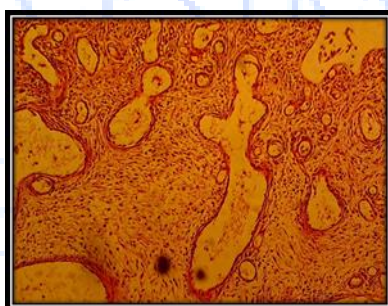


Figure: (11): Cervical polyp tissue with H&E stains (10X).

3.3: Expression of *HOXB4* in cervix

3.3.1: *HOXB4* gene expression for the three groups with cervical patients

This figure (12) indicated expression of *HOXB4* in cervical cancer and benign tumors patients in comparison with control group. Expression the means of *HOXB4* (0.69 ± 0.12) was reported in 15 cases of cervical cancer of out of 45 cervical patients, (0.91 ± 0.08) in 20 cases of cervical benign of out of 45 cervical patients, (1.07 ± 0.14) in 10 cases of cervical control of out of 45 cervical patients (Figure3.29).

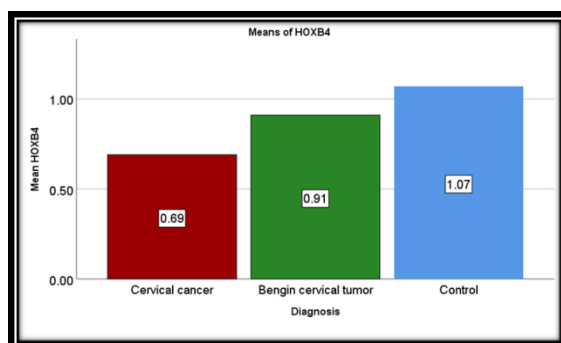


Figure (12): Means of *HOXB4* gene expression for the three groups with cervical patients.

These results showed the expression the means *HOXB4* of three groups (cancer, benign and control) of cervical patients. Patients with cervical cancer had the lowest mean (0.69 ± 0.12) and the highest value mean expression *HOXB4* for patients with cervical control (1.07 ± 0.14) among the three groups. Results of (10) demonstrated that *HOXB4* was markedly downregulated in cervical cancer, also proved functioned as agrowth-inhibition role in cervical cancer, *HOXB4* obviously suppressed cervical cancer cell proliferation and tumorigenic potential, *HOXB4* induced cell cycle arrest at the transition from the G0/G1 phase to the S phase and loss of *HOXB4* promoted cervical cancer cell growth both in vitro and in vivo.

3.3.2. Mean difference in *HOXB4* gene expression between cervical cancer and control patients.

Table (15) indicated the means expression *HOXB4* of cervical cancer patients in comparison with control group. The mean expression of *HOXB4* (0.69 ± 0.12) was recorded with Standard deviation (0.12) in cervical cancer patients and mean expression of *HOXB4* (1.07 ± 0.14) was recorded with Standard deviation (0.14) in cervical control patients group Table (15) Mean difference in *HOXB4* gene expression between cervical cancer and control patients.

Table(15): Mean difference in *HOXB4* gene expression between cervical cancer and control patients.

Group	Mean	Standard deviation	P-value
Cervical cancer	0.69	0.12	<0.001
Control	1.07	0.14	

These results shows the values *HOXB4* expression a significant difference (P-value <0.001) between patients with cervical cancer and control group. It appeared that *HOXB4* expression means in cervical cancer patients (0.69 ± 0.12) had a lower than control group (1.07 ± 0.14). This result is agreed with the result of (10) who indicated that the qRT-PCR showed that compared with the normal cervix, the expression of *HOXB4* in randomly selected SCC patient tissues was reduced, these data indicated that compared with the normal cervix, the *HOXB4* expression was downregulated in cervical cancer. Also, (43) found that gene expression microarray data revealed a significant alteration of 12 out of 39 HOX cluster members among cervical cancer cases, in comparison to the histopathologically normal controls. In addition, López-Romero *et al.*(2015)²⁵⁴ showed that *HOXB4* was overexpressed in many cancers including cervical cancer.

3.3.3. Mean difference in *HOXB4* gene expression between cervical benign tumor and control patients.

Table (16) indicated the means *HOXB4* expression of cervical benign patients in comparison with control group. The mean *HOXB4* expression (0.91 ± 0.08) was recorded with standard deviation (0.08) in cervical benign patients and mean *HOXB4* expression (1.07 ± 0.14) was recorded with Standard deviation (0.14) in cervical control patients group Table:(16)Mean difference in *HOXB4* gene expression between cervical benign and control patients.

Table (16): Mean difference in *HOXB4* gene expression between cervical benign and control patients.

Group	Mean	Standard deviation	P-value
Cervical benign tumor	0.91	0.08	<0.001
Control	1.07	0.14	

These results shows even the mean *HOXB4* expression for patients with benign cervical tumors (0.91 ± 0.08) was slightly lower than control group (1.07 ± 0.14), this difference was statistically significant (P-value <0.001). This *HOXB4* gene expression in cervical benign tumor was studied for

the first time in Iraq by PCR analysis and there are no previous studies in modern years and it needs more studies

3.3. 4. Mean difference in HOXB4 gene expression between cervical cancer and benign tumor patients.

Table (17) indicated the means expression HOXB4 of cervical cancer tumor patients in comparison with cervical benign tumor patients. The mean expression of HOXB4 (0.69 ± 0.12) was recorded with standard deviation (0.12) in cervical cancer patients and mean expression of HOXB4 (0.91 ± 0.08) was recorded with standard deviation (0.08) in cervical benign patients Table (17). Mean difference in HOXB4 gene expression between cervical cancer and benign tumor patients.

Table (17): Mean difference in HOXB4 gene expression between cervical cancer and benign tumor patients.

Group	Mean	Standard deviation	P-value
Cervical cancer	0.69	0.12	<0.001
Cervical benign tumor	0.91	0.08	

These results regarding the values mean expression HOXB4 difference between cervical cancer patients and benign tumor patients was significant (P-value >0.001). Patients with cervical cancer mean expression HOXB4 (0.69 ± 0.12) had a lower mean than expression HOXB4 those diagnosed with benign tumor (0.91 ± 0.08). The results of (44) found that mechanism studies showed that HOXB4 negatively regulated Wnt/ β -catenin signaling pathway. Mutations in the Wnt pathway and its components are responsible for a variety of growth-related pathologies and cancers. Transcription factors of the HOX family have an important role in numerous cellular processes, including cell growth by regulating various signaling pathways, such as the Wnt/ β -catenin signaling pathway.

Conclusions

In this study, we endeavored to understand the differential gene expression patterns of *HOXB4* across varying states of cervical tissue health, namely, cervical cancer (CC), benign tumors, and normal controls. Our results have provided compelling insights into the potential roles these gene play in colorectal tumorigenesis and disease progression.

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