ORIGINAL ARTICLE

Prognostic significance of annexin II , human epididymis protein (HE-4) and claudin in endometrial carcinoma

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ABSTRACT

Introduction: Endometrial cancer (EC) is one of the most prevalent malignant tumors of the female reproductive system worldwide. Annexins are membrane binding proteins with important role in tumor development and progression. Human Epididymis Protein (HE-4) is a novel marker for gynecolgical tumors. Claudins are proteins of tight junction category playing an important role in cell adhesion and tumor spread.
Material and methods: Seventy blocks of paraffin-embedded tissues of endometrial carcinoma cases. Immunohistochemical evaluation of Annexin II , HE- 4 and Claudin-7 staining was performed. Clinical follow-up to all cases was done every three months.
Results: Positive Annexin II,HE-4 expression were observed in 88.6% and 77.1% of EC respectively. Significant correlation was found between expression of both Annexin II and HE-4 and FIGO stage, decreased both overall and disease free survival rates. Positive Claudin-7 expression was observed in 40% of EC, with significant correlation with high grade only, however, no correlation with other clinical parameters or survival analysis was detected.
Conclusion: Annexin II, HE-4 and Claudin-7 are prognostic factors for endometrial carcinoma and could be used in molecular targeted therapy.
Key Words: Endometrial carcinoma, AnnexinII, HE-4, Claudin-7, IHC, Prognosis

1. INTRODUCTION

Endometrial cancer is one of the most prevalent malignant tumors of the female reproductive system worldwide and considered one of the most causes of cancer-related deaths among women.[1] Most patients with advanced stage of endometrial carcinoma have limited treatment options,[2] therefore, early diagnosis and treatment is an important approach for improving the survival rate of patients with endometrial carcinoma.[3]

Prognostic assessment of EC relies mostly upon degree of tumor differentiation, International Federation of Gynecology and Obstetrics (FIGO) staging and histologic type of EC.[4] However, these prognostic factors alone are not adequate for predicting tumor recurrence, so management of endometrial carcinoma is in need of new markers that may predict relapse of disease in high hazard cases by methods of immunohistochemistry.[5] Annexin II is a calcium-dependent phospholipid-binding protein that is is primarily expressed in human endothelial cells, mononuclear cells, macrophages

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and marrow cells with multiple cellular functions including endocytosis, exocytosis, and cellular adhesion.[6] Annexin II plays different roles in cancer growth and progression through involvement in cell signaling pathways, proliferation, invasion, metastasis and tumor neovascularization.[7,8]

It is overexpressed in a variety of human malignancies as breast, cervical and pancreatic carcinomas, so it may have an important role in the targeted therapy of these cancers.[9] Human epididymis protein-4 (HE-4) is a member of protease inhibitors family that is normally present in the epithelial cells of the epididymal duct. HE-4 is considered as a novel marker for gynecolgical tumors especially ovarian and endometrial malignancies.[10] Recent studies showed that the HE-4 not only expressed in gynecolgical tumors, but also in other tumors.[11] Many studies suggested the value of (HE-4) in the prognosis of endometrial carcinoma.[12,13] Not only a prognostic marker but also HE-4 might be assistant in the diagnosis of endometrial cancer.[14]

Tight junctions comprise proteins, such as occludin, claudin, and junctional adhesion molecules. Destruction of tight junctions between cells are important to the invasion and metastasis of malignant tumor cells.[15]

Claudin-7 is an important member of the claudin family and plays an important role in maintaining tight junction integrity, epithelial cell polarity, and ion permeability between cells.[16] Abnormal Claudin-7 expression has been closely related to the development of various malignant tumors where it is abnormally regulated either downregulated[17,18] or overexpressed.[19]

The downregulation of Claudin-7 is considered an important step in tumorigenesis and metastasis.[20] In endometrial carcinoma, the major role of Claudin-7 is to regulate the invasive processes of malignant cells and epithelial-mesenchymal transition, so can provide a potential strategy for targeted therapy.[20] In the current study, immunoexpression of Annexin II, HE-4 and Claudin-7 in endometrial carcinoma was studied and their relation to patient survival and tumor relapse.

2. Patients and methods

This study was performed in Gynecology and Obstetrics, Pathology and Clinical Oncology departments, Faculty of medicine, Zagazig University. Seventy blocks of paraffin-embedded endometrial tissues were selected from the archives of the Pathology department , during the period between 2015 and 2019, where they were diagnosed as endometrial carcinoma. Five patients with (stage I) underwent radical surgery, thirty nine patients with (stage II) experienced radical medical procedure and postoperative (adjuvant) radiotherapy, eight(8) inoperable patients with (stage III) had gotten radiotherapy and 18 patients with (stage IV) had gotten chemotherapy. Cases were analyzed by two pathologists and reviewed according to the World Health Organization (WHO) evaluating framework and arranged by criteria of the International Federation of Gynecolology and Obstetrics (FIGO). Immunoexpression of the three markers in all endometrial samples was assessed, and their correlation with clinico-pathological and prognostic parameters. Follow up, survival, recurrence rate, and therapeutic response were also detected. All cases were followed up every three months in Medical Oncology Department and in Clinical Oncology and Nuclear Medicine Department, Faculty of medicine, Zagazig University.

2.1 Immunohistochemistry

The streptavidin–biotin immunoperoxidase method was used. Segments of 3-5 μm from the formalin-fixed-paraffin-blocks were cut and mounted on positively charged slides. Dewaxing in xylene and drying in ethanol. Antigen recovery by warming in autoclave (10 mM, pH 6.0) for about 20 minutes. The endogenous peroxidase action was obstructed with 6% hydrogen peroxide for fifteen minutes in methanol. Positive and negative controls were utilized. Concentrations of essential antibodies against HE-4 and Annexin II utilized were 1:100 (Abcam, Rabbit polyclonal to HE-4 GeneTex (1-877-436-3839) and 1:1200 (Abcam, Rabbit polyclonal to Annexin II Catalog Number GTX101902), respectively. Primary anti-Claudin-7 antibodies concentration was 1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA). The specimens were incubated for two hours at 37°C.[21]

2.2 Immunohistochemical scoring

Annexin II and HE-4 positive cells were recognized by brown coloured granules on the cytoplasm. The percentage of stained cells was calculated as follow: the proportion of positive cells < 5% was scored as 0, 5%–25% as 1, 26%–50% as 2, 51%–75% as 3, and as 4 if staining > 75%.[22]

For evaluation of Claudin-7: membranous or cytoplasmic brown-staining was considered positive with the following criteria: 0 :negative expression; 1: weak expression (1%-15%); 2, moderate expression (16%-49%); and 3, strong expression (50%-100%). All slides were independently scored by two pathologists.[23]

2.3 Statistical analysis

Constant factors were expressed as the mean ± SD & median (range), and the categorical variables were communicated as a number (rate). Continuous variables were checked for ordinariness by utilizing Shapiro-Wilk test. Mann Whitney

U test was used to compare between two groups of non-normally distributed variables. Kruskal Wallis H test was used to compare between more than two groups of normally distributed variables. Percent of categorical variables were compared using Pearson's Chi-square test or Fisher's exact test when was appropriate. Disease Free Survival (DFS) was calculated as the time from date of surgery to relapse or the most recent follow-up in which no relapse was detected. Overall Survival (OS) was calculated as the time from diagnosis to death. Stratification of DFS and OS was done according to Anexin II, HE-4 and Claudin-7 expression in tumor cells. These time-to-event distributions were evaluated utilizing the technique for Kaplan-Meier plot, p-esteem < .05 was viewed as significant. All insights were performed utilizing SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA) and MedCalc windows (MedCalc Software bvba 13, Ostend, Belgium).

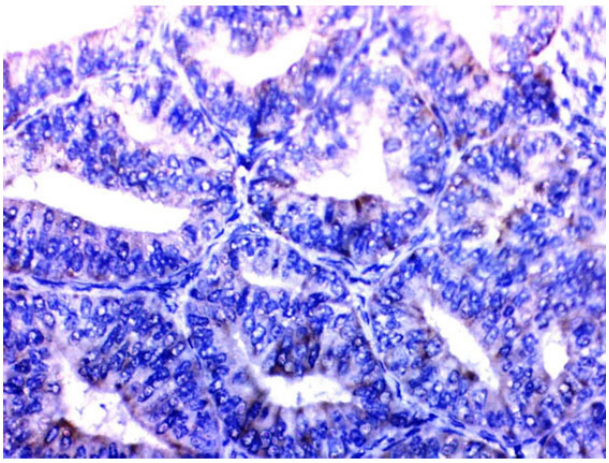

Figure 1. Negative Claudin staining in low grade endometrial carcinoma × 400

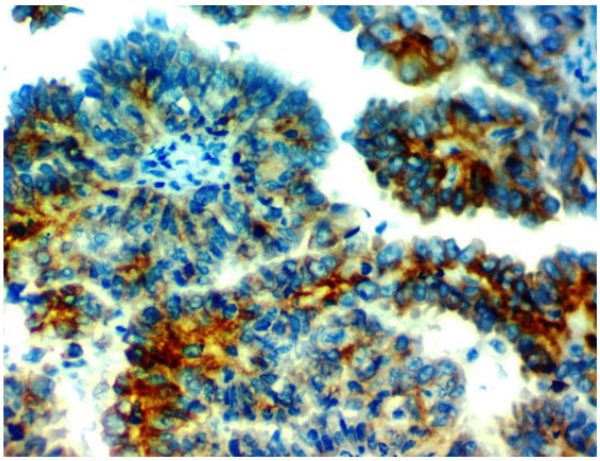

Figure 2. Weak positive Claudin staining in moderate grade endometrial carcinoma × 400

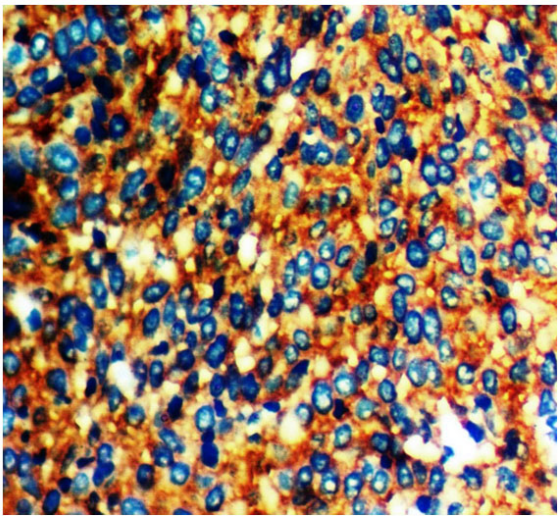

Figure 3. Highly positive Claudin staining in high grade endometrial carcinoma × 400

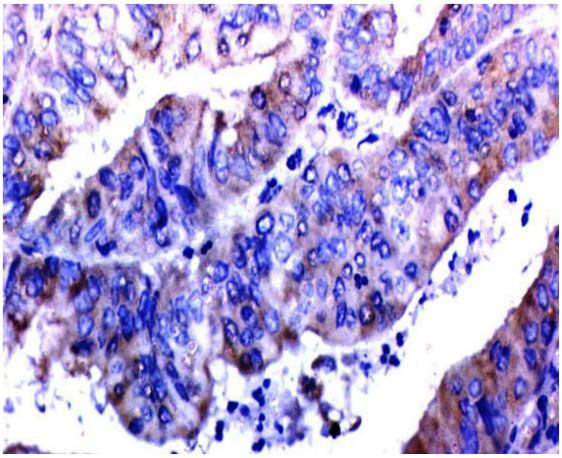

Figure 4. Weak positive HE4 staining of endometrial carcinoma × 400

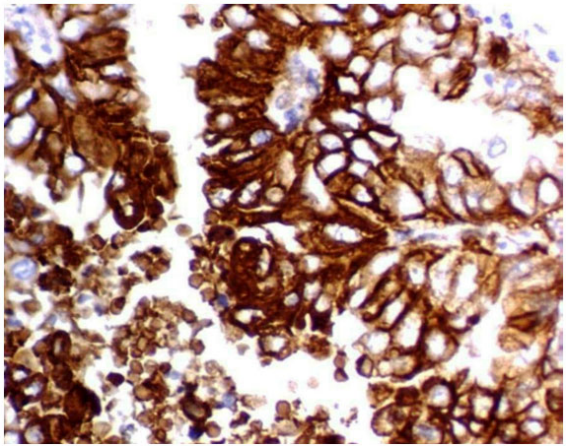

Figure 5. Highly positive HE4 membranous staining of endometrial carcinoma × 400

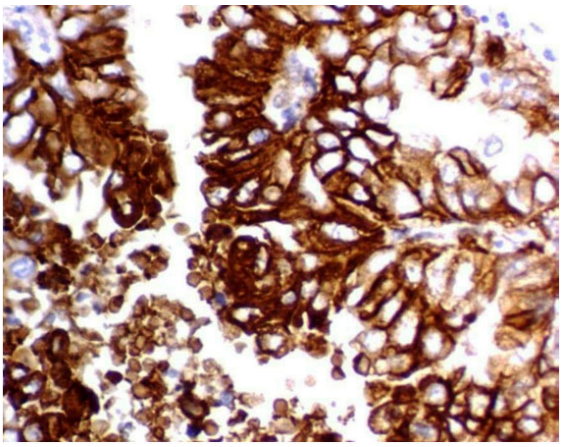

Figure 6. Positive Annexin 2 staining in endometrial carcinoma × 400

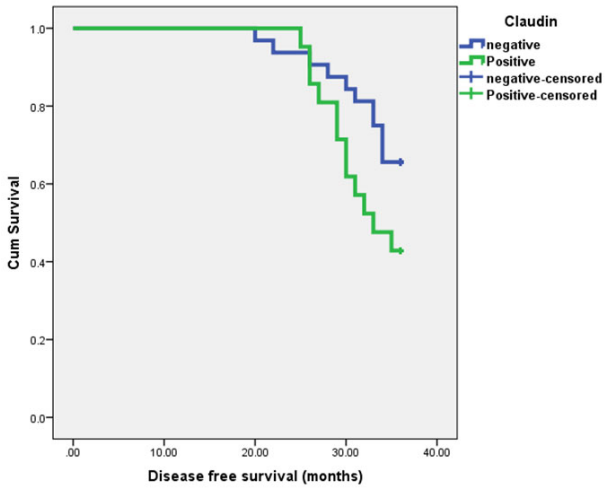

Figure 7. Kaplan Meniere graph showing disease free survival in cases with claudin positive and negative.

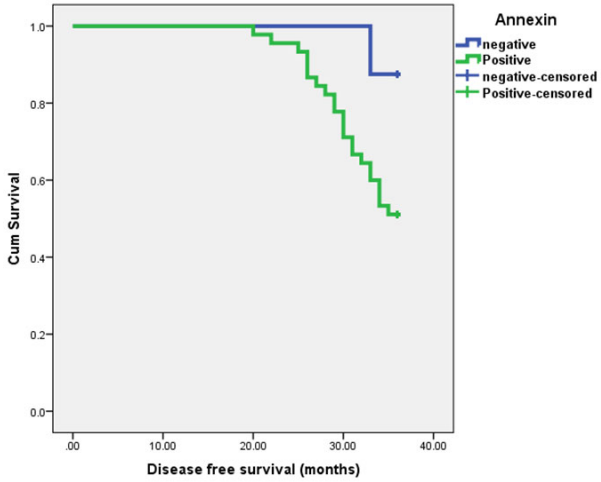

Figure 8. Kaplan Meniere graph showing disease free survival in cases with annexin positive and negative

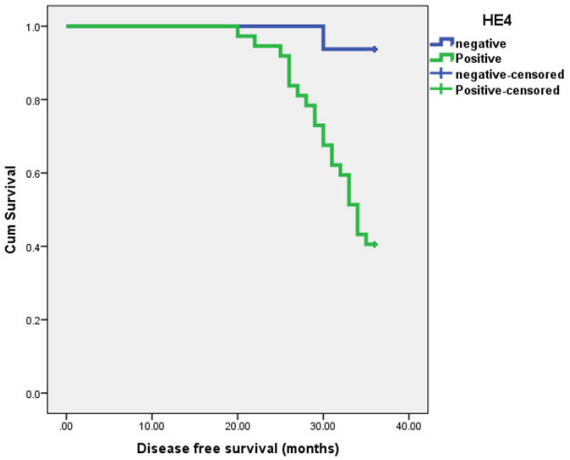

Figure 9. Kaplan Meniere graph showing disease free survival in cases with He4 positive and negative

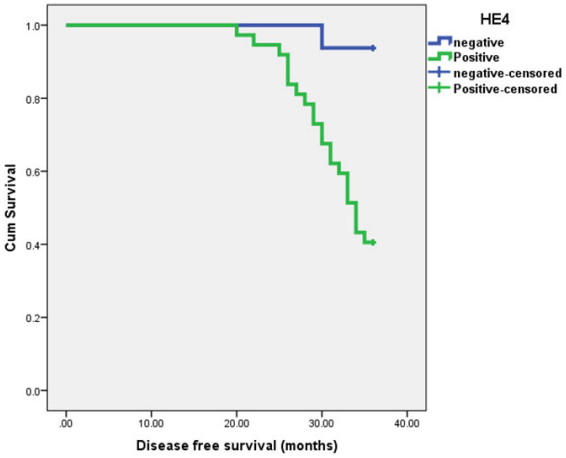

Figure 10. Kaplan Meniere graph showing overall survival in cases with claudin positive and negative.

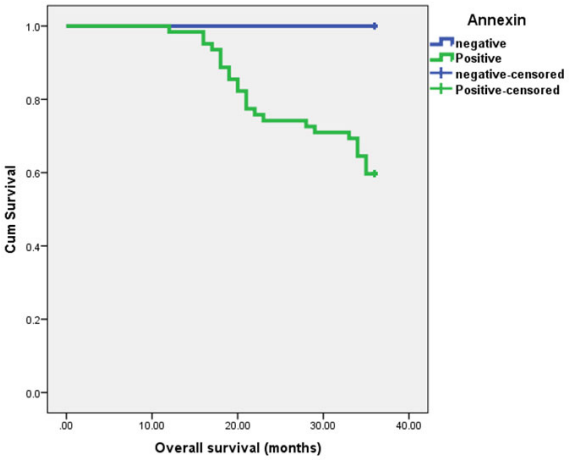

Figure 11. Kaplan Meniere graph showing overall survival in cases with Annexin positive and negative.

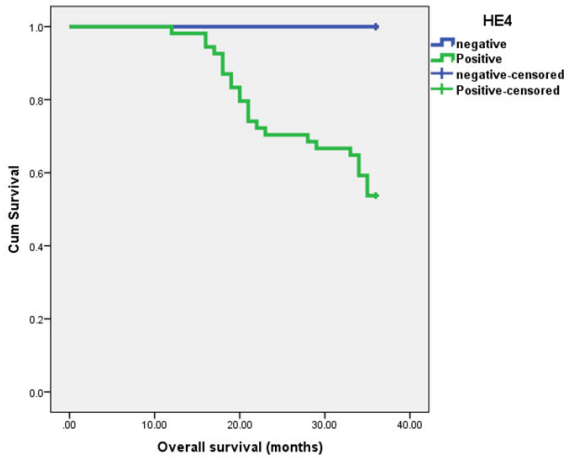

Figure 12. Kaplan Meniere graph showing overall survival in cases with He4 positive and negative

3. Results

3.1 Patients' characteristics: (see Table 1)

The age of the patients was (45-65) years. The pathological surgical staging was performed by the 2009 International Federation of Gynecology and Obstetrics framework as follows: 15 cases as stage I; 21 cases as stage II, 16 cases as stage III,; and 18 cases as stage IV.

3.2 Relation between clinicopathological features, and immunostaining of Annexin II, HE-4 and Claudin-7 in the studied patients: (see Tables 2, 3, 4), (Figures 1-6)

Positive Annexin II expression was observed in 88.6% and positive HE-4 staining was observed in 77.1% of tissue samples. Both Annexin II and HE-4 was found to be significantly associated with both higher FIGO stage and increased rate of lymph node metastasis. However, no association was found between each of these markers and tumor grade. Claudin-7 expression was observed in 40% of cases. There is a significant association found between Claudin-7 positive expression and high grade cases $p < .001$ and lymph node metastasis ($p = .097$), however, no association was found between Claudin-7 expression and tumor stage.

3.3 Correlation between Annexin II, HE-4 and Claudin-7, (see Table 5)

Both Annexin II and HE-4 positive expressions was significantly associated with each other, $p = .013$. There is no association between any of these two markers and Claudin-7 positive staining.

Table 1. Clinicopathological features, immunohistochemical markers and outcome of the studied patients

Variable	N = 70	%
Grading		
I	30	42.9
II	27	38.6
III	13	18.5
Lymph node		
Absent	36	51.4
Present	34	48.6
Staging		
I	15	21.4
II	21	30
III	16	22.9
IV	18	25.7
Relapse n = 53		
Absent	30	56.6
Present	23	43.4
Death		
Absent	45	64.3
Present	25	35.7
Annexin II		
Negative	8	11.4
Positive	62	88.6
HE-4		
Negative	16	22.9
Positive	54	77.1
Claudin-7		
Negative	42	60
Positive	28	40
Age		
X ±SD	58.11 ±9.29	
Range	25 - 69	
Disease free survival (53)	33.17 ±4.17	
X ±SD	20 - 36	
Range		
Overall survival		
X ±SD	31.69 ±7.3	
Range	12 - 36	

3.4 Association between disease relapse, patient survival and markers immunoexpression: (see Table 6, 7, Figures 7-12)

We analyzed the disease free survival and overall survival of endometrial carcinoma patients using Kaplan-Meier method Regarding disease free survival (DFS) and the 3-years overall survival (OS). A significant difference was observed between patients with negative Annexin II and HE-4 expression and those with positive expression, with respect to DFS in Annexin II where the mean DFS for negative patients was significantly longer than that of positive patients (35.6 versus 32.7 months, $P < .001$) and 3-year OS was 36 versus 31.1 months, $p < .001$ respectively.

Table 2. Relation between clinicopathological features and immunohistochemical staining for Annexin II in the studied patients

Variable	Annexin II		Test	P
	Negative(8) No(%)	Positive (62) No(%)		
Grading				
I	6 (20)	24 (80)		
II	2 (7.4)	25 (92.6)	4.286‡	.117
III	0 (0)	13 (100)		
Lymph nodes				
Absent	8 (22.2)	28 (77.8)	Fisher	.005*
Present	0 (0)	34 (100)		
Staging				
I	6 (40)	9 (60)		
II	2 (9.5)	19 (90.5)	16.559‡	.001**
III	0 (0)	16 (100)		
IV	0 (0)	18 (100)		
Age				
Mean ± SD	59.88 ± 4.67	57.89 ± 9.72	0.567∞	.573
Range	49 - 64	25 -69		
Disease free survival				
Mean ± SD	35.63 ±1.06	32.73 ± 4.37	3.846∞	< 0.001**
Range	33 - 36	20-36		
Overall survival				
Mean ± SD	36 ± 0	31.13 ± 7.58	5.06∞	< 0.001**
Range	36 − 36	12 − 36		

Table 3. Relation between clinicopathological features and immunohistochemical staining for HE- 4 in the studied patients

Variable	HE-4		Test	*P*
	Negative (16)	Positive (54)		
Grading				
I	7 (23.3)	23 (76.7)		
II	8 (29.6)	19 (70.4)	2.402	.301
III	1 (7.7)	12 (92.3)		
Lymph nodes				
Absent	14(38.9)	22 (61.1)	Fisher	.001**
Present	2 (5.9)	32 (94.1)		
Staging				
I	9 (60)	6 (40)		
II	5 (23.8)	16 (76.2)	18.054	< .001**
III	2 (12.5)	14 (87.5)		
IV	0 (0)	18 (100)		
Age				
Mean ±SD	59.06 ± 4.7	57.83 ± 10.3	0.673	.504
Range	49 - 65	25 -69		
Disease free survival				
Mean ±SD	35.63 ±1.5	32.11 ± 4.51	4.032	< .001**
Range	30-36	20-36		
Overall survival				
Mean ± SD	36 ± 0	30.41 ± 7.88	5.219	< .001**
Range	36 - 36	12 - 36		

Table 4. Relation between clinicopathological features, and immunohistochemical staining for Claudin-7 in the studied patients

Variable	Claudin-7		Test	*P*
	Negative (42)	Positive (28)		
Grading				
I	27 (90)	3 (10)		
II	15 (55.6)	12 (44.4)	30.972‡	< .001**
III	0 (0)	13 (100)		
Lymph nodes				
Absent	25 (69.4)	11 (30.6)	2.755‡	.097
Present	17 (50)	17 (50)		
Staging				
I	12 (80)	3 (20)		
II	13 (61.9)	8 (38.1)	5.916‡	.116
III	6 (37.5)	10 (62.5)		
IV	11 (61.1)	7 (38.9)		
Age				
Mean ±SD	57.43 ±9.14	59.14 ±9.58	-0.754∞	.453
Range	25 - 68	26 - 69		
Disease free survival				
Mean ±SD	33.78 ±4.21	32.24 ±4.04	1.326∞	.191
Range	20 - 36	25 - 36		
Overall survival				
Mean ±SD	31.83 ±7.23	31.46 ±7.53	0.206∞	.838
Range	16 - 36	12 - 36		

Table 5. Correlation between Claudin-7, Annexin II and HE- 4 immunostaining in the studied patients

Variable	Claudin-7		*p*	Annexin II		*p*	HE-4		*p*
	Negative (42)	Positive (28)		Negative (8)	Positive (62)		Negative (16)	Positive (54)	
Claudin-7									
Negative				7 (16.7)	35 (83.3)	.132	10 (23.8)	32 (76.2)	.816
Positive				1 (3.6)	27 (96.4)		6 (21.4)	22 (78.6)	
AnnexinII									
Negative	7 (87.5)	1 (12.5)	.132				5 (62.5)	3 (37.5)	.013*
Positive	35 (56.5)	27 (43.5)					11 (17.7)	51 (82.3)	
HE- 4									
Negative	10 (62.5)	6 (37.5)	.816	5 (31.2)	11 (68.8)	.013*			
Positive	32 (59.3)	22 (40.7)		3 (5.6)	52 (94.4)				

Table 6. Mean disease survival in both arms (Annexin II positive and negative)

Measure	Negative	Positive	*P*
Mean disease free survival	35.63	32.73	.001**

Table 7. Mean disease survival in both arms (HE4 positive and negative)

Measure	Negative	Positive	*P*
Mean disease free survival	35.63	**32.11**	.001**

Table 8. Mean disease free survival in both arms (Claudin-7 positive and negative)

Measure	Negative	Positive	*P*
Mean disease free survival	33.78	**32.34**	.077

Table 9. Mean overall survival in both arms (Annexin positive and negative)

Measure	Negative	Positive	*P*
Mean overall survival	36	**31.13**	.001**

Table 10. Mean overall survival in both arms (HE4 positive and negative)

Measure	Negative	Positive	*P*
Mean overall survival	36	**30.41**	< .001**

Table 11. Mean overall survival in both arms (Claudin-7 positive and negative)

Measure	Negative	Positive	*P*
Mean overall survival	31.83	**31.46**	0.410

In addition, a significant difference between negative HE4 and positive HE-4 patients with respect to OS, where the mean OS for negative patients was significantly longer than the mean OS for positive patients (35.63 versus 32.11 months, $P < .001$) and the 3-year OS was 36 versus 30.41 monthes respectively $P < .001$). There was no significant difference between negative and positive Claudin expression. ($P = .191$ and $P = .838$ respectively).

4. Discussion

In the current study, positive Annexin II and HE-4 expressions were observed in 88.6% and 77.1% of tissue samples respectively. Both Annexin II and HE-4 found significantly associated with both higher FIGO stage and increased rate of lymph node metastasis. However, no association was found between each of these markers and tumor grade.

These results are in accordance with another study by Deng et al.[24] who reported that the Annexin II, HE-4 levels increased in both endometrial carcinoma and atypical hyperplasia than normal endometrium, and overexpression of both markers was significantly associated with lymph nodal metastasis and myometrial invasion.

Significant correlation between Annexin II and HE-4 was found. Annexin II was identified as an HE-4 interacting protein. This interaction promoted tumor invasion and metastasis in endometrial carcinoma as proved by Deng et al.[24]

A significant difference was observed between patients with negative Annexin II and HE-4 expression and those with positive expression, with respect to DFS, OS where the mean DFS, OS for negative patients was significantly longer than that of positive patients.

Current study results are close to Alonso et al.[25] who found that Annexin II can be used as a potential marker of endometrial carcinoma recurrence, through the promotion of endometrial carcinoma metastasis and in line with the recent studies by Stiekema et al.[26] who observed that HE-4 was an independent prognostic factor for both DFS and OS in patients with EC.

Angioli et al.[27] announced that preoperative HE-4 levels were higher in patients who developed relapsing disease contrasted with patients without relapse. Brennan et al.[28] also showed that HE-4 was a sensitive and specific predictor of recurrent disease.

In a study made by Capriglione et al.[29] they reported that HE-4 level was higher in recurrent cases than cases without recurrence.

In the current study, Claudin-7 expression was observed in 40% of cases, down regulated in endometrial carcinoma than non-neoplastic tissues, with significant correlation between Claudin-7 expression and high grade cases ($p < .001$) and lymph node metastasis ($p = .097$). This finding is consistent with another study made by Li et al.[23] who documented that the ectopic expression of Claudin-7 significantly regulated the proliferation and invasion of endometrial carcinoma cells.

Lu et al.[30] analyzed the expression of Claudin-7 in normal lung tissues and lung carcinomas, and found that Claudin-7 is weak or absent in lung carcinoma.Claudin-7 level is also downregulated and significantly associated with histological grade and stage in breast and pancreatic carcinomas.[31,32]

In their study, Xu et al.[33] reported that Claudin-7 expression was significantly lower in colorectal carcinoma (CRC) than in surrounding non-neoplastic tissues and Claudin-7 expression was positively correlated with CRC differentiation. In addition, they found that the rates of positive Claudin-7 expression was decreased in lymphatic metastases and liver metastasis.

Decreased Claudin-7 expression correlated with EC differentiation; so that Claudin-7 could inhibit the occurrence of EC. Claudin-7 expression was significantly decreased in lymphatic metastases, suggesting that Claudin-7 downregulation may be related to the invasion and metastasis of EC.

5. Conclusion

Annexin II, HE-4 could be used as prognostic factors that can predict tumor recurrence and could be of benefit in endometrial carcinoma molecular targeted therapy. Claudin-7 is a tumor suppressor gene for EC, therefore, it is expected to be an early diagnostic marker and a new therapeutic target for EC.

Conflicts of Interest Disclosure

The authors declare no conflict of interest.

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