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FOXO1 Downregulation Correlates with Progression of Esophageal Intraepithelial Neoplasia

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Abstract ***Background:*** Forkhead transcription protein 1 (FOXO1) is an important transcriptional regulator of cell proliferation and is considered essential for tumor growth and progression. However, the function of FOXO1 in esophageal cancer remains unclear. Esophageal squamous intraepithelial neoplasia (ESIN) has been widely recognized as a precursor lesion for esophageal squamous cell carcinoma (ESCC). Early detection offers the best prognosis for esophageal squamous cell carcinoma. The differentiation of squamous dysplasia from reactive change and the classification of ESIN into high-grade (HGIEN) or low-grade (LGIEN) are sometimes subjective and challenging. In this study, we sought to evaluate the FOXO1 expression in endoscopically biopsied esophageal cases and compare its expression with those of p AKT1, Ki67 and p53 to detect where is the lesion stand in the scale from normal mucosa to HGIEN in order not to miss prone cancer cases. ***Methodology and results:*** Immunohistochemical staining was performed on 99 formalin-fixed, paraffin embedded blocks of endoscopic esophageal samples. Out of them, 44 samples were ESIN and 33 samples were ESCC, further, for comparison, 22 samples of reactive hyperplastic, non-dysplastic esophageal mucosa. The current study showed a significant stepwise decrease in the FOXO1 expression as well as increased expression of p AKT1, ki67 and P53 from the progression of LGIEN to HGIEN to ESCC. HGIEN and ESCC strongly associated with low FOXO1 expression. On the other hand, higher expression of p AKT1, ki67 and p53 was more associated with HGIEN and ESCC (all are P: 0.001). ***Conclusions:*** This study demonstrated stepwise under-expression of FOXO1 in the progression of esophageal squamous carcinogenesis so that immunohistochemical assessment of this marker might provide a useful adjunct tool for differential diagnosis between LGIEN and HGIEN as well as could be used in targeting therapy.

Keywords: *Forkhead transcription protein 1 _ Esophageal Squamous intraepithelial neoplasia _ Esophageal squamous cell carcinoma_p AKT1_ Ki67_ P53*

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1. Introduction

Esophageal squamous cell cancer (ESCC) is one of the most common lethal tumors in the world due to late diagnosis, local relapse, distant metastasis, and resistance to adjuvant therapy [1]. Esophageal cancer precursor lesions early detection in the tiny endoscopic samples is inconclusive in most of the cases and therefore, new pathological diagnostic markers are needed to enhance the ability of early detection of early squamous neoplasia of the esophagius [2].

Esophageal squamous intraepithelial neoplasia (ESIN) defined as non-invasive cytological or architectural alterations that may lead to the development of invasive squamous cell carcinoma [3].

The development of ESCC is a multistep process, progressing from normal squamous epithelium to noninvasive precursor lesions, initially containing low-grade intraepithelial neoplasia (LGIEN), and then containing high-grade intraepithelial neoplasia (HGIEN), carcinoma in situ (CIN) and eventually to invasive carcinoma [4]. It is important to distinguish LGIEN from HGIEN because the managements are different. As local treatment such as endoscopic mucosal resection (EMR) or local surgical treatment is recommended for HGIEN, follow up only is recommended for LGIEN [5]. Unlike the squamous intraepithelial lesions in the uterine cervix and oral cavity, in which several biomarkers have been well characterized as useful tools for diagnosing the precursor lesions for ESCC, no biomarkers have been developed for ESIN so far [6].

The Forkhead transcription factors (FOXO) family includes three functionally related members, FOXO1a/FKHR, FOXO3a/ FKHRL1, and FOXO4/AFX [7]. FOXO1 is an important downstream target of phosphatidylinositol- 3-kinase (PI3K)/AKT signaling pathway, regulates cellular homeostasis by maintaining cell proliferation, apoptosis, and viability in normal cells. Though, the function, and regulation of FOXO1 is well documented in many cancers, the molecular mechanism of its regulation in esophageal cancer is largely unknown [8].

Deregulation of FOXO1 has been shown to promote cell proliferation and tumorigenesis in the prostate, breast, and endometrial cancer cells [9,10]. FOXO1 has become a major target in preventing tumorigenesis [11,12]. However, the relationship between the clinical significance and FOXO1 expression in esophageal carcinogenesis has not been established.

Akt (also called protein kinase B) is a serine/threonine protein kinase activated by a variety of growth factors, via the phosphatidylinositol 3-kinase (PI3K) pathway, and plays a role in tumorigenesis by inhibiting apoptosis and mediating cell proliferation [13,14]. Phosphorylation achieves a full activity of Akt at Thr308 and Ser473[15]. Phospho-Akt (p-Akt) is a potent promoter of cell survival as it antagonizes and inactivates various components of the apoptotic cascade, such as members of the forkhead transcription factor family [16]. Besides, Akt has been implicated in regulating metastasis, which is an important process in cancer development. P-AKT1 (Thr308) is upregulated in primary tumors and cell lines [17,18].

The aim of this study to evaluate endoscopically biopsied esophageal cases subjected to immunohistochemical analysis of FOXO1 immunoreactivity and compare its expression with those of p AKT1, Ki67 and p53 to detect where is the lesion stand in the scale from normal mucosa to HGIEN in order not to miss prone cancer cases.

2. Material and Methods

2.1. Case Selection

This study is a retrospective one included 99 endoscopic esophageal samples. Out of them, 44 samples were ESIN, and 33 samples were ESCC, further, for comparison, 22 samples of reactive hyperplastic, non-dysplastic esophageal mucosa were selected from cases used for exclusion of Barrett's esophagus.

All studied cases are formalin-fixed, paraffin embedded blocks and their related data regarding age and sex were retrieved from surgical files of Pathology Department, Tanta University and private laboratories, Egypt within the period from January 2014 to September 2015.

This study was approved by the ethics committee of Faculty of Medicine, Tanta University, Egypt. The collected material was archived paraffin blocks only, number coded and not identified by the patient's name. There was no contact with patients and hence informed consent was not required.

2.2. Histopathological Evaluation

Sections were cut at 4 µm and routinely stained with hematoxylin and eosin. All fragments of esophageal squamous mucosa were classified as LGIEN or HGIEN and invasive ESCC according to 2000 World Health Organization classification [19]. According to this classification, CIS was not separated from HGIEN, because they may have the same clinical implications and a similar risk to progress to invasive ESCC. Of the studied 99 cases, 18 cases were LGIEN, 26 cases were HGIEN, 33 cases were ESCC and 22 cases were reactive hyperplastic, non-dysplastic esophageal mucosa.

2.3. Immunohistochemistry Assay

The antibodies used in the study are mentioned in the Table 1, including their clones, manufacturers, antigen retrieval method, buffer pH for the retrieval, positive control, incubation time, and temperature.

Table 1. Antibodies used in this study

Antibody to	Clone	Company	Raised in	Positive control	Antigen retrieval method	Dilution and incubation time
FOXO1	D-19 polyclonal	Santa Cruz Biotechnology, Santa Cruz, CA, USA	Rabbit	Vaginal rhabdomyosarcoma	Citrate buffer, pH 6.0	1:200 Overnight,4ºC
P AKT1 (phospho S473)	EP2109Y Monoclonal	Abcam, Cambridge, UK	Rabbit	Cervical carcinoma	Citrate buffer pH 9.0	1:100 One hour, room temperature
Ki67	Mib-1 Monoclonal	DAKO, Glostrup, Denmark	Mouse	Lymph node germinal center	Citrate buffer pH 6.0	1:100 Overnight, 4ºC
P53	DO-7 Monoclonal	DAKO, Glostrup, Denmark	Mouse	Colon carcinoma	Citrate buffer pH 6.0	1:1000 Overnight, 4ºC

2.4. Immunohistochemistry Procedure

The avidin-biotin-peroxidase complex method was used for the immunostaining of FOXO1, p AKT1, Ki67, and P53. In brief, after dewaxing, inactivating endogenous peroxidase activity and blocking cross-reactivity with normal serum (Vectastain Elite Kit; Vector, Burlingame, CA, USA), the sections were incubated for 60 minutes at room temperature or overnight at 4C, with a diluted solution of the primary antibody. Location of the primary antibodies was achieved by subsequent application of a biotinylated anti-primary antibody, an avidin-biotin complex conjugated to horseradish peroxidase, and diaminobenzidine (Vectastain Elite Kit, Vector, Burlingame, CA). The slides were counterstained with

hematoxylin. Negative controls were established by replacing the primary antibody with PBS and normal mouse or rabbit serum.

2.5. Assessment of Immunostaining

The positive immunostain of FOXO1, Ki-67, and p53 was exclusively nuclear staining. The positive immunostain of p AKT1 included combined nuclear and cytoplasmic stain. The cytoplasmic or nuclear stain alone was considered as a negative stain. The immunostained sections were evaluated at a high power of standard light microscope with a 40 objective. The degree of immunostaining based on both the proportion of positively stained tumor cells and the intensity of staining. The proportion of tumor cells was scored as follows:

0 (no positive tumor cells or positive cells in the basal layer),

1 (<10% positive tumor cells or involving 1/3 of the thickness of the epithelium),

2 (10–50% positive tumor cells or involving 2/3 of the thickness of the epithelium),

3 (>50% positive tumor cells or involving more than 2/3 of the thickness of the epithelium).

The intensity of staining was graded according to the following criteria: 0 (no staining); 1 (weak staining=light yellow), 2 (moderate staining=yellow brown) and 3 (strong staining=brown).

The staining index (SI) was calculated as staining intensity score × proportion of positive tumor cells. Using this method of assessment, the evaluation of FOXO1 expression was scored using the SI, as 0, 1, 2, 3, 4, 6 and 9. The optimal cutoff value for FOXO1 that used in the study was identified previously by Li et al., [20] who used SI score of ≥ 4 to define the high FOXO1 expression and ≤ three as low expression of FOXO1. The scores for p Akt1, Ki67, and P53 immunostaining were also performed using the method mentioned above.

2.6. Statistical Analysis

Statistical presentation and analysis of the present study were conducted, using the mean, standard deviation, and chi-square test, person correlation by SPSS (version 15.0; SPSS Inc., Chicago, Illinois, USA) software. Significant differences were considered at $p < 0.05$.

3. Results

3.1. Clinicopathological Characteristics of Reactive Esophageal Mucosa, LGIEN, HGIEN, and ESCC

The clinicopathological characteristics of LGIEN (18 cases), HGIEN (26 cases), and ESCC (33 cases), reactive esophageal mucosa (22 cases) are shown in Table 2.

Mean ages were 50-72 years for LGIEN, 51-72 years for HGIEN, 53-76 years for ESCC and 51-72 for reactive samples.

As regards sex, males were more frequent in all studied cases; their percentages were represented as 77.78%, 69.23%, 85.85% and 72.73% in LGIEN, HGIEN, ESCC and reactive samples respectively.

LGIEN was located equally in the middle or lower esophagus (44.44%), meanwhile, HGIEN, ESCC and reactive samples were more frequent located in the middle esophagus (57.69, 60.61 and 45.45 % respectively).

Table 2. Clinicopathological characteristics of LGIEN, HGIEN, ESCC and Reactive esophageal mucosal samples

Average			LGIEN	HGIEN	ESCC	Reactive
Age[a]	Range		50-72	51-72	53-76	51-72
	Mean±SD		61.61±6.79	63.34±6.91	67.33±5.37	60.36±7.10
Sex	Female	N	4	8	5	6
		%	22.22	30.77	15.15	27.27
	Male	N	14	18	28	16
		%	77.78	69.23	84.85	72.73
	Total	N	18	26	33	22
		%	100.00	100.00	100.00	100.00
Location	lower	N	8	9	9	9
		%	44.44	34.62	27.27	40.91
	middle	N	8	15	20	10
		%	44.44	57.69	60.61	45.45
	upper	N	2	2	4	3
		%	11.11	7.69	12.12	13.64
	Total	N	18	26	33	22
		%	100.00	100.00	100.00	100.00

a P- value: 0.001

3.2. Immunoexpression of FOXO1, p AKT1, Ki67 and p53 in Studied Endoscopic Samples

FOXO1, pAKT1, ki67 and p63 expression in reactive esophageal squamous Epithelium, different precursor lesions, and ESCC was illustrated in Table 3 and diagram (1). FOXO1 was significantly highly expressed in LGIEN and reactive esophageal mucosa. Meanwhile, AKT, p53, and p16 were significantly mainly expressed in ESCC and HGIEN.

Furthermore, the level of high FOXO1 expression decreased gradually in the transformation from non-dysplastic epithelium in 14 reactive esophageal mucosa cases (77.87%) (Figure 1. A2), 14 LGIEN (63.64%) (Figure 1. B2), 4 HGIEN (15.38%) (Figure 1. C2), to invasive ESCC (0.00%) (Figure 1. D2). This result was statistically significant.

pAKT1, ki67, and p53 were significantly overexpressed in HGIEN and ESCC. High p Akt1 expression increased gradually from non-dysplastic epithelium in 5 reactive esophageal mucosa cases (22.73%) (Figure 1. A3), and 2 LGIEN (11.11%) (Figure 1. B3), to 14 HGIEN (53.85%) (Figure 1. C3), and 24 invasive ESCC (72.73%) (Figure 1. D3).

Ki67 was highly expressed in 16 ESCC (48.49%) (Figure 1. D4) and 4 HGIEN (15.38%) (Figure 1. C4).Whereas Ki67 expression was negative in 19 reactive esophageal mucosa cases (86.36%) (Figure 1. A4) and 13 LGIEN (72.22%), Five LGIEN cases (27.78%) were Ki67 positive but exhibiting low positivity (Figure 1. B4).

P53 expression was negative in 12 reactive esophageal mucosa cases (68.18%) (Figure 1. A5) and 15 LGIEN (66.67%) (Figure 1.B5), on the other hand, it was demonstrated in 13 HGIEN cases (50%) and 14 ESCC cases (42.42%), exhibiting low positivity. High p53 positivity was found in 4 HGIEN (15.38%) (Figure 1. C5) and 9 ESCC (27.27%) (Figure 1. D5).

According to these results, there was a significant decrease in FOXO1 expression in ESEN progression. On the contrary, the pattern was the opposite for p AKT1, p53, and Ki-67 staining expression in the studied cases (p:0.001).

Table 3. Immunoexpression of FOXO1, p AKT1, Ki67 and p53 in 99 studied samples

			Type							
			LGIEN	**HGIEN**	**ESCC**	**Reactive**	**Total**			
Foxo1	**Negative**	**N**	2	4	24	3	33	**Chi-square**	**X^2**	**71.564**
		%	11.11	15.38	72.73	13.64	33.33			
	Low	**N**	2	18	9	5	34			
		%	11.11	69.23	27.27	22.73	34.34			
	High	**N**	14	4	0	14	32		**P-value**	0.001
		%	77.78	15.38	0.00	63.64	32.32			
pAKT1	**Negative**	**N**	4	2	4	4	14	**Chi-square**	**X^2**	27.804
		%	22.22	7.69	12.12	18.18	14.14			
	Low	**N**	12	10	5	13	40			
		%	66.67	38.46	15.15	59.09	40.40			
	High	**N**	2	14	24	5	45		**P-value**	0.001
		%	11.11	53.85	72.73	22.73	45.45			
Ki67	**Negative**	**N**	13	6	2	19	40	**Chi-square**	**X^2**	23.597
		%	72.22	23.08	6.06	86.36	40.40			
	Low	**N**	5	16	15	3	39			
		%	27.78	61.54	45.45	13.64	39.39			
	High	**N**	0	4	16	0	20		**P-value**	0.001
		%	0.00	15.38	48.49	0.00	20.20			
P53	**Negative**	**N**	12	9	10	15	46	**Chi-square**	**X^2**	38.176
		%	66.67	34.62	30.30	68.18	46.46			
	Low	**N**	6	13	14	7	40			
		%	33.33	50.00	42.42	31.82	40.40			
	High	**N**	0	4	9	0	13		**P-value**	0.000
		%	0.00	15.38	27.27	0.00	13.13			
Total		**N**	18	26	33	22	99			
		%	100.00	100.00	100.00	100.00	100.00			

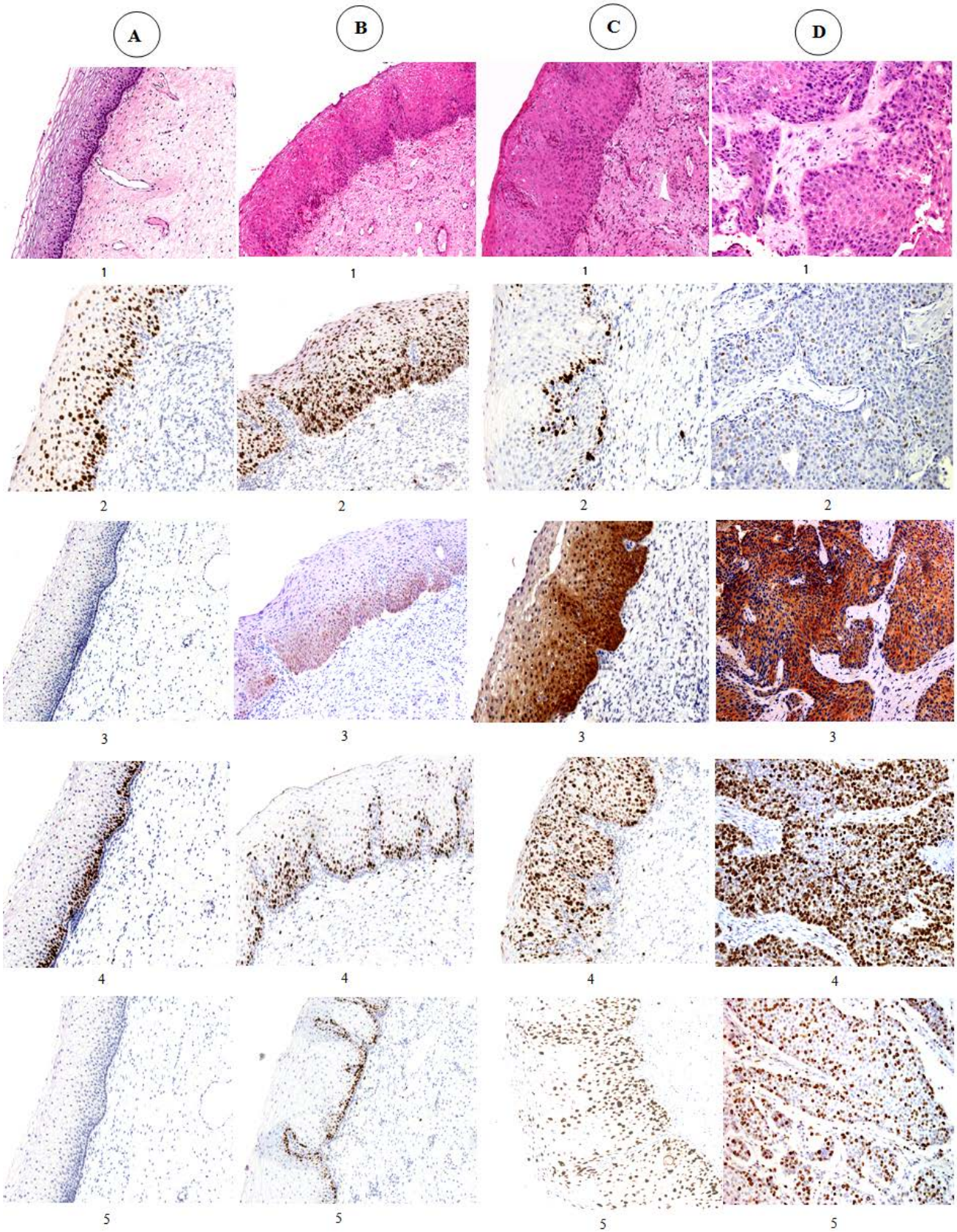

Figure 1. Immunoexpression of FOXO1, p AKT1, Ki67, and p53

A (1-5), H& E Reactive esophageal mucosa (1), high FOXO1 staining score 9 (2), negative staining patterns of p AKT1, KI67 and p53 (3-5),

B (1-5), H& E LGIEN (1), high FOXO1 staining score 9 (2), low p AKT1 & Ki67 staining score 1 (3-4), negative staining of P53 (5),

C (1-5), H & E HGIEN (1), negative FOXO1 staining score (2), high p AKT1 staining score 6 (3), high Ki67 staining score 9 (4), and p53 staining score 6 (5).

D (1-5), H& E ESCC (1), negative FOXO1 staining (2), high p AKT1, Ki67 staining score 9 and p53 staining score 6 (3-5).

(H&E and immunohistochemistry, ×200).

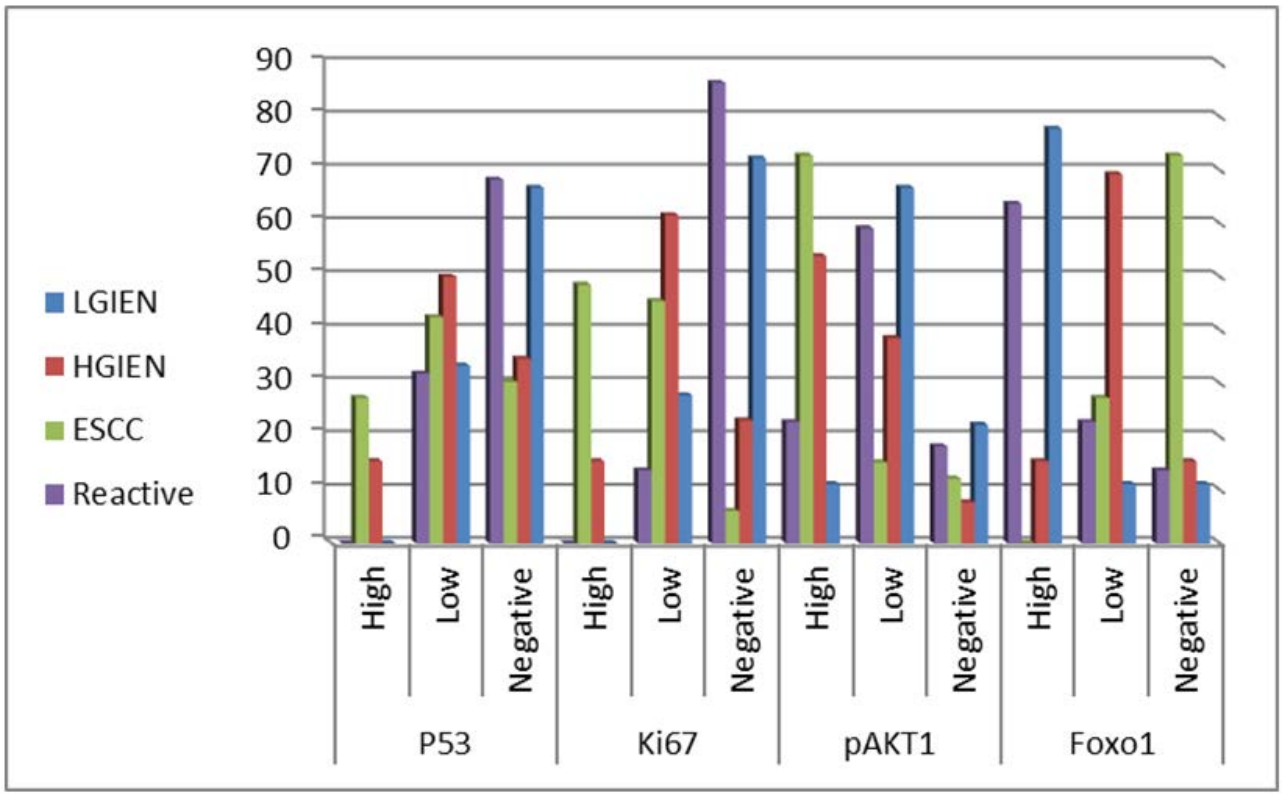

Diagram 1. Immunoexpression of FOXO1, p AKT1, Ki67 and p53 in 99 studied samples

3.3. Correlation between FOXO1 Expression and other Markers

The Pearson's correlation coefficient (*r*) was calculated to measure the correlating behavior among the different studied markers in both neoplastic esophageal lesions and reactive esophageal mucosa. Briefly, it was found that FOXO1 was significantly in inverse correlation with the other three markers. On the other hand, there was a significant positive correlation between p Akt1, p53 and Ki67 as shown in the Table 4 ($p < 0.001$). Thus, down regulation of FOXO1 correlated with the progression of ESCC and might be a late event in the multistep pathogenesis of ESCC.

Table 4. Correlation between FOXO1 expression and other markers in studied samples

Correlations		Foxo1	pAKT1	P53
pAKT1	R	-0.343		
	P-value	0.001		
P53	R	-0.304	0.557	
	P-value	0.002	<0.001	
Ki67	r	-0.454	0.402	0.612
	P-value	<0.001	<0.001	<0.001

4. Discussion

Esophageal squamous cell cancer is one of the most aggressive tumors. Even though significant advances in the therapeutic approach to this disease, the mortality rate of esophageal cancer remained almost with a 5-year survival rate of 10% to 20% [21]. The difficulty in diagnosing patients of ESCC at an early stage predisposes to their poor prognosis. The risk of developing invasive cancer in LGIEN is low; however HGIEN and CIS is likely to progress to invasive cancer and thus requires early treatment [22,23]. Therefore, it would be of great clinical benefit to early detect where the lesion is standing in the scale from normal mucosa to HGIEN. Some studies speculate that biological events that account for the malignancy and development of ESCC can be introduced as prognostic biomarkers in precursor lesions [24,25,26].

To evaluate the difference between low risk and potentially malignant esophageal lesions, FOXO1, p AKT1, Ki67 and P53 expression was studied on 99 different esophageal samples. In the present study, we did not separate carcinoma in situ from HGIEN as 2000 World Health Organization classification suggested because they may have the same clinical implications and a similar risk to progress to invasive carcinoma [19]. However, it is important to distinguish LGIEN from HGIEN because the managements are different.

The current study showed a significant stepwise decrease in the FOXO1 expression as well as the increase in the expression of p AKT1, ki67 and p53 from the progression of LGIEN to HGIEN to ESCC. There is a strong association between lower FOXO1 expression and HGIEN and ESCC. On the other hand, higher expression of p AKT1, ki67 and p53 is more associated with HGIEN and ESCC. For our knowledge, this is the first study to evaluate the role of FOXO1 expression in esophageal lesions.

In the current study, FOXO1 expression was negative or low positive in ESCC and HGIEN in comparison to reactive esophageal mucosa and LGIEN. This finding is in agreement with results of other studies in different cancers

[12,27,28]. Additionally, the level of high FOXO1 expression decreased gradually in the transformation of the reactive epithelium (77.87%), LGIEN (63.64%), HGIEN (15.38%), to invasive ESCC (0.00%). From these results, it was hypothesized that downregulation of FOXO1 correlated with the progression of ESCC and could be a delayed step in the pathogenesis of ESCC.

Zhang et al. [29], studied FOXO1 expression in cervical squamous dysplasia, they had shown that FOXO1 expression is elevated in low dysplasia and is further decreased in severe dysplasia and cancer cases. These results are concordant with this work, which displays the stepwise loss of FOXO1 expression in HGIEN and ESCC.

Maekawa et al. [30] stated that the expression of FOXO1 in non-small cell lung cancer is related to early cancer status, suggesting that FOXO1 expression may be an appropriate marker for predicting prognosis. They recommend that inhibition of the Akt/FOXO1 signaling pathway may contribute to novel molecular targeted treatment strategies for prevention of esophageal cancer development.

On the contrary, p Akt1 expression increased gradually from the reactive esophageal epithelium (22.73%) and LGIEN (11.11%) to HGIEN (53.85%) and invasive ESCC (72.73%). This result was in agreement with some studies have demonstrated the overactivation of AKT in many human solid tumors and hematological malignancies [19,31,32].

The prognostic values of p-AKT1 have been investigated in several malignancies [13,15,17,21]. Interestingly, many studies have shown that activation of AKT1 associated with poor prognosis. PI3K/AKT signaling pathway has been linked to cancer progression and has a crucial role in tumor development. FOXO1 is an important downstream effector of this pathway. Previous studies have demonstrated FOXO1 as a direct substrate of Akt and an association between these two proteins has been observed in some human cancers including glioblastoma [33], lung cancer [34] and breast cancer [35], but no such association has been observed in prostate cancer. Besides some authors suggested that p AKT1 could be used potentially as a target for anticancer therapeutics in ESCC [21].

Ki67 was positive in the basal layer of the esophageal epithelium in many cases, reflecting active cell division in the lower layer. While Ki67 was negative mainly in the upper layer of tissue samples in the reactive esophageal mucosa (86.36%) LGIEN (72.22%) but was highly positive in ESCC (48.49%) and HGIEN (15.38%), suggesting active cell division in the upper layer of the esophagus in HGIEN.

This result is in agreement with results of Wang et al. [2] that reported the Ki67 expression of the esophageal dysplasia was significantly higher than that of normal epithelium and the Ki67 increased with an increase in the grade of dysplasia.

In the present study, p53 overexpression was detected in 50 % and 42.42% of HGEIN and ESCC respectively. This result suggests there was a stepwise increment in p53 expression, in line with other results of p AKT1 and ki67. These findings were in agreement with the results of Nakayama et al. [25], Taniere et al. [36] and Parenti et al. [37]. This could be used as a useful method for histopathologic evaluation of the risk for progression to invasive carcinoma in esophageal epithelial lesions .

Interestingly, FOXO1 expression was also in negative correlation with p AKT1, ki67 and P53 expression in all studied cases. These findings suggest that the down-regulation of FOXO1 expression plays a significant role in repression of esophageal cancer development.

Some studies have tried to combine different markers, such as in cervical intraepithelial neoplasia, to increase diagnostic accuracy and concordance among different pathologists [29,38]. Although using more than one marker may slightly increase sensitivity or specificity in some cases, the increased cost and complicated strategy may limit its clinical usage. In this study, we recommend FOXO1 to be used as an objective and useful method for histopathologic evaluation of the risk for progression to invasive carcinoma in esophageal intraepithelial lesions, resulting in adequate therapy.

This study indicates that the underexpression of FOXO1 and the overexpression of P AKT1, Ki67, and p53, have an important role during the early stages of esophageal squamous carcinogenesis.

In conclusion, this study showed stepwise under-expression of FOXO1 in the progression of esophageal squamous carcinogenesis, so that immunohistochemical assessment of this marker might provide a useful adjunct tool for differential diagnosis between LGIEN and HGIEN as well as could be used in targeting therapy.

Conflicts of Interest

There is no conflict to be declared

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