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Research Article

Immunohistochemical Expression of SIX1 in Papillary Thyroid Carcinoma

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Abstract

Background: Thyroid cancer (TC) is the most common type of malignant endocrine cancer and it's ranked as the 10th most common cancer worldwide, and its incidence increases, due to improvement of diagnostic procedures. Papillary thyroid cancer (PTC) is the most frequent type of differentiated thyroid carcinoma. In Egypt PTC subtype represents 85% of all TC cases. SIX1 is expressed in a wide range of normal and neoplastic tissues and it has different pathways in tumorigenesis in different tissues. SIX1 is upregulated in human thyroid cancer cell and promoted cell proliferation and invasion. **Aim of the work:** this study was conducted to research the association between SIX1 expression and the clinicopathological features of PTC. **Material and Methods:** Immunohistochemical staining of SIX1 was conducted on 50 randomly chosen tissue specimens of PTC 34 (68%) were classic PTC and 16 (32%) were follicular subtype, that was done by using the avidin biotin-peroxidase complex method with diaminobenizidine (DAB) chromogen detection system. **Results:** High SIX1 expression was detected in 36% of cases. SIX1 expression showed statistically significant correlation with tumor focality (P= 0.003), lymph node metastasis (P= 0.002), tumor grade (P= 0.002), advanced tumor stage (P= 0.01) assessed by pT staging system, tumor necrosis (P= 0.002), lymphovascular, perineural invasion, and extra thyroidal extension (P= 0.002), (P= 0.01) and (P= 0.001) respectively. **Conclusions:** SIX1 expression is significantly associated with poor prognostic factors and thus can be used as a prognostic indicator for PTC patients.

Key words: SIX1, PTC, Immunohistochemistry.

Introduction

Thyroid cancer (TC) is ranked as the 10th most common cancer worldwide [1], In Egypt, it's considered the 5th most common cancer in Egypt [2] and its incidence increases, due to improvement of diagnostic procedures leading to early detection [3]. In children, about 2–6% out of pediatric malignancies are considered TC, so it is the most common endocrine cancer in them [4]. Papillary thyroid cancer (PTC) is the most predominant subtype of TC, and it's considered 80% to 85% of the thyroid malignant diseases [5.6.7]. In Egypt PTC subtype represents 85% of all TC cases [2].

Transcription factors (TFs) are regulatory proteins that coexist on genomic regions and interact with each other which can lead to the regulation of gene expression. Gene regulation induced by TFs depends on their ability to bind DNA elements and to form protein complexes [8]. Cancer has been developed by alteration in TFs via various direct mechanisms including chromosomal translocations, gene amplification or deletion, point mutations and alteration of expression [9]. SIX1 is a member of TF family having pivotal roles in organ development and differentiation, acting to regulate the expression of different proteins that are involved in embryogenesis and in disorders like cancer [10], it can act as oncogene through induction of epithelial mesenchymal transition (EMT), also activates the transforming growth factor (TGF) signal

transduction so promoting the tumor progression [11].

In PTC, SIX1 promotes the proliferation and invasion of malignant cells by activation of signal transducers and activators of transcription 3 (STAT3) signaling, and also provide significant protection against apoptosis via activation of the classical STAT3 signaling [12], it was found to be upregulated in PTC and was associated with tumor size and nodal metastasis, and it was proven that SIX1 overexpression contributes to tumor initiation but still with unclear diagnostic or prognostic value [13].

Material and Methods

Tissue specimens

The present study comprised 50 randomly selected cases of primary PTC. We have several clinicopathological variables including: patient age, sex, tumor site, tumor size, tumor focality, tumor histological subtypes, tumor grade, lymphovascular invasion (LVI), perineural invasion (PNI), tumor necrosis, lymph node status, extrathyroidal extension (ETE) and tumor stage. Tumor type and grade were submitted to WHO classification of thyroid neoplasms, 5th edition [14], and tumor stage was estimated by AJCC 8th Edition/ TNM classification system for differentiated thyroid carcinoma (DTC) [15]. (see table 1).

The mean age ± standard deviation (SD) of the studied cases was 42.02 ± 14.6, ranged from 18-80 years. The age of the patients was classified into two main groups; < 55 years and ≥ 55 years according to the median age of patients included in this study. Forty-two cases over fifty (84%) of patients were < 55 years of age and 8/50 (16%) of patients ≥ 55 years. Regarding sex of patients in our study, 6/50 cases (12%) were males and 44/50 cases (88%) were females. Regarding the tumor site, 21/50 (42%) of tumors were found in the right lobe as primary tumor site, 14/50 (28%) were located in the left lobe, and 15/50 (30%) of tumors were bilateral.

With respect to tumor size in maximum dimension, with a mean size ± SD of 2.31 ± 1.46, the minimum size was 0.5 cm, and maximum size was 6 cm. Tumors were categorized into 3 groups: the first group included 28/50 (56%) tumors which were ≤ 2 cm, the second group included 17/50 (34%) tumors which were > 2 and ≤ 4 cm, and the third group included 5/50 (10%) tumors > 4 cm. Concerning tumor focality, we had 33/50 (66%) patients with unifocal tumor, while 17/50 (34%) had multifocal tumor.

Regarding the histological subtypes of PTC, 34/50 (68%) cases were classic PTC, and 16/50 (32%) cases were follicular subtype. In the present study, PTC cases with low grade features were 40/50 (80%), while cases of PTC with high grade features were 10/50 (20%) of our cases.

Concerning LVI, 14/50 (28%) cases were positive, and 36/50 (72%) cases were negative. Regarding PNI, 8/50 (16%) cases were positive, and 42/50 (84%) cases were negative. At the time of primary diagnosis, 12/50 (24%) of cases had LNM, whereas 38/50 patients (76%) had negative lymph node for malignancy.

Nine cases (18%) of PTC in our study, showed ETE, and 41 cases (82%) were negative for ETE. Regarding tumor necrosis, 10/50 (20%) cases showed tumor necrosis, and 40/70 (80%) cases showed no tumor necrosis. According to AJCC 8th Edition/ TNM classification system, 21/50 cases (42%) of tumors were pT1, while 16/50 cases (32%) of tumors were pT2, 10/50 cases (20%) of tumors were pT3, and 3/50 cases (6%) were pT4.

Table 1: Clinicopathological features for patients with PTC (n=50)

Clinicopathological Data	NO.	Percent
Age (years)		
< 55	42	84%
≥ 55	8	16%
Sex		
Male	6	12%
Female	44	88%
Site		
Right	21	42%
Left	14	28%
Bilateral	15	30%
Size (cm)		
≤ 2	28	56%
>2 - ≤ 4	17	34%
> 4	5	10%
Focality		
Unifocal	33	66%
Multifocal	17	34%
Histologic Subtype		
Classic PTC	34	68%
Follicular subtype	16	32%
Grade		
PTC with low grade features	40	80%
PTC with High grade features	10	20%
LVI		
Negative	36	72%
Positive	14	28%
PNI		
Negative	42	84%
Positive	8	16%
Nodal Status		
Negative	38	76%
Positive	12	24%
ETE		
Negative	41	82%
Positive	9	18%
Tumor Necrosis		
Negative	40	80%
Positive	10	20%
Tumor Stage		
PT1	21	42%
PT2	16	32%
PT3	10	20%
PT4	3	6%

-LVI: Lymphovascular Invasion
-PNI: Perineural Invasion
-ETE: Extrathyroidal Extension

Immunohistochemistry

Sections with Five µm in thickness were used on glass slides that were positive charged for immunohistochemistry of SIX1 primary antibodies using the complex of avidin biotin-peroxidase method and diaminobenzidine (DAB) chromogen detection system. Firstly, we deparaffined and rehydrate tissue sections on the positive charged slides. Second step the endogenous peroxidase was blocked by flooding in a 3% solution of hydrogen peroxide and left for half an hour. Then, Antigen retrieval was done by submerging the slides in citrate buffer solution with pH concentration 6, twice (10 minutes each) at 750-W. To block non-specific background staining, the slides were exposed to UV block. The primary antibody SIX1 was then added and tissue sections were incubated for an hour at room temperature (dilution 1:500). We get rid of the excess reagent and the slides were then flooded gently with buffer solution for 5 minutes. After that Secondary biotinylated antibody was added for each slide for 30 minutes. DAB substrate and chromogen. The Positive control for SIX1 was normal human endo-cervical glands.

Scoring of Immunostaining

Cytoplasmic or nuclear SIX1 expression is considered positive. The immunohistochemical scores were obtained as multiplication of staining intensity (scored from 0–3) by the positive cell percentage (scored from 1–4). The intensity of SIX1 protein expression was scored as: 0 (no staining); 1 (weak staining, light yellow color); 2 (moderate staining, pale brown); or 3 (strong staining, chocolate brown). The percentage of positive cells was scored as: 1 (0-25%); 2 (26%–50%); 3 (51%–75%); 4 (76%–100%). Cut-off value for high SIX1 protein is score of > 6, while low expression of the SIX1 protein is when the final score is ≤ 6 [12]. and the expression was divided into 2 categories; low expression and high expression. For statistical purposes, negative cases are included among low expressions and considered as one category.

Statistical analysis

Statistical analysis was conducted using the IBM SPSS 20.0 statistical package software (IBM; Armonk, New York, USA).

Results

In the present study, SIX1 expression in all cases was expressed only in the cytoplasm, while SIX1 positive staining was not detected in any of adjacent non-cancerous thyroid tissue (ANCT) (0/32). On comparing SIX1 expression between non-tumor and PTC tissue; an obvious significant difference was found ($p< 0.001$), with 18/50 (36%) revealed high marker expression as shown in **(figure1),** whereas 32/50 cases (64%) exhibited -ve/low cytoplasmic SIX1 expression, as shown in **(figure 2).**

No statistically significant association was found between SIX1 expression and patient's age, sex, tumor site, tumor size, and the histologic subtypes (P= 0.3, P= 0.1, P= 0.06, P=0.09, and P= 0.4) respectively. In the current study, SIX1 expression showed statistically significant association with tumor multifocality (P= 0.003), LNM (P= 0.002) **(Figure 3)**, tumor with high grade features (P= 0.002), advanced tumor stage (P= 0.01) assessed by pT staging system, tumor necrosis (P= 0.002) **(Figure 4)**, LVI (P= 0.002) **(Figure 5)**, PNI (P= 0.01) **(Figure 6)** and ETE (P= 0.001) **(Figure 7)**. See table (2).

Table (2): Association between SIX1 expression and clinicopathological features for patients with PTC (n=50)

	SIX1		p value
	Low expression (N=32)	High expression (N=18)	
Age (y)			
<55 y	28 (66.7%)	14 (33.3%)	0.3
≥ 55 y	4 (50%)	4 (50%)	
Sex			
Male	2 (33.3%)	4 (66.7%)	
Female	30 (68.2%)	14 (31.8%)	0.1
Tumor site			
Right lobe	16 (76.2%)	5 (23.8%)	0.06
Left lobe	10 (71.4%)	4 (28.6%)	
Bilateral	6 (40%)	9 (60%)	
Histological subtypes			
Classic PTC	21 (61.8%)	13 (38.2%)	0.4
Follicular subtype PTC	11 (68.8%)	5 (31.2%)	
Tumor size			
≤ 2	19 (67.9%)	9 (32.1%)	0.09
>2 - ≤ 4	12 (70.6%)	5 (29.4%)	
>4	1 (20%)	4 (80%)	
Tumor Focality			
Unifocal	26 (78.8%)	7 (21.2%)	**0.003**
Multifocal	6 (35.3%)	11 (64.7%)	
Nodal status			
Negative	29 (76.3%)	9 (23.7%)	**0.002**
Positive	3 (25%)	9 (75%)	
Tumor grade			
PTC without high grade features	30 (75%)	10 (25%)	**0.002**
PTC with high grade features	2 (20%)	8 (80%)	
Tumor Stage			
pT1	17 (81%)	4 (19%)	
pT2	12 (75%)	4 (25%)	**0.01**
pT3	3 (30%)	7 (70%)	
pT4	0	3 (100%)	
Tumor Necrosis			
Negative	30 (93.75%)	10 (55.5%)	**0.002**
Positive	2 (6.25%)	8 (44.5%)	
LVI			
Negative	28 (77.8%)	8 (22.2%)	**0.002**
Positive	4 (28.6%)	10 (71.4%)	
PNI			
Negative	30 (71.4%)	12 (28.6%)	**0.01**
Positive	2 (25%)	6 (75%)	
ETE			
Negative	31 (75.6%)	10 (24.4%)	**0.001**
Positive	1 (11.1%)	8 (88.9%)	

**** P - value <0.0 are considered statistically significant according to Chi-Square test.***

On comparing SIX1 expression in primary tumor and their corresponding metastatic lymph node tissues, a concordance rate of 80% in SIX1 expression was found; where high SIX1 expression in both was detected in 3/4 (75%) cases and negativity or low expression in both was detected in 5/6 (83.3%) cases. However, as shown in table (3), there was no significant difference in SIX1 expression between them (P= 0.07).

Table (3): Comparison between the expression of SIX1 in the primary tumor and corresponding lymph node metastasis:

primary tumor Vs corresponding lymph node metastasis		Primary Tumor SIX1 Expression		P value
		Low	High	
Nodal SIX1 Expression	Low expression	5 (83.3%)	1 (16.7%)	**0.07**
	High expression	1 (25%)	3 (75%)	

**** P - value <0.05 are considered statistically significant.***
**** Test of significance is: spearman correlation test.***

In our study, we examined SIX1 expression in each subtype of PTC with comparison to the different clinicopathological data.
In CPTC, in regard to the age of patients, patients were classified into <55 and ≥ 55 y, most cases 29/34 (85.3%) were < 55 year. Twenty nine out of thirty-four cases (85.3%) were females. In regarding to the size of tumor, most cases 20/34 (58.8%) were with tumor size ≤ 2. Bilaterality was detected in 12/34 (35.3%) and multifocality was recorded in 12/34 (35.3%) of cases. Concerning LVI, 9/34 (26.5%) of cases had positive LVI, while only 6/34 (17.6%) of cases that showed positive PNI. Positive LNM was detected in 8/34 (23.5%). ETE, was positive in 5/34 (14.7%) of cases. Eight cases (23.5%) of CPTC were positive for tumor necrosis. In respect of tumor grade 8/34 (23.5%) of cases with high grade. Twenty-five cases (73.5%) were with stage pT (1 and 2).

In FV-PTC, in regard to the age of patients, 13/16 (81.3%) of cases were <55 years old. Fifteen cases (93.8%) were females. In regarding to the size of tumor, 8/16 (50%) with tumor size ≤ 2 and the other half of cases were with tumor size >2 - ≤ 4. Bilaterality was detected in only 3/16 (18.8%) of cases, and multifocality was found in 5/16 (31.3%) of cases. Positive LVI was recorded in 5/16 (31.3%) of cases. Only two cases (12.5%) were positive for PNI. While in nodal status, 4/16 (25%) of cases were positive LNM. ETE was also positive in (25%) of cases. In regarding to tumor necrosis only 2/16 (12.5%) of cases were positive. As well, in respect of, tumor grade (12.5%) of cases were high grade. While 12/16 (75%) of cases with stage pT (1 and 2).

High SIX1 expression was observed in 38.2% (13/34) of CPTC versus 31.3% (5/16) in FVPTC cases. On comparing SIX1 expression in CPTC and FVPTC in relation to their different clinicopathological data, the statistical analyses revealed significant positive associations between high SIX1 expression and tumor site, multifocality, tumor with high grade features, tumor necrosis in CPTC cases (P= 0.04, P= 0.02, P= 0.03 and P= 0.03, respectively), and significant positive associations between high SIX1 expression and LNM, advanced tumor stage, LVI, and ETE in FVPTC cases (P= 0.003, P= 0.007, P= 0.01 and P= 0.003, respectively). (Table 4).

Table (4): Comparison between the expression of SIX1 in classic and follicular subtypes of PTC with clinicopathological data:

	Classic N=34			**Follicular N=16**		
	Low expression N=21	**High expression N=13**	**p-value**	**Low expression N=11**	**High expression N=5**	**p-value**
Age range						
<55	19 55.9%	10 29.4%	0.3	9 56.25%	4 25%	0.7
≥55	2 5.9%	3 8.8%		2 12.5%	1 6.25%	
Sex						
Male	1 2.9%	4 11.7%	0.05	1 6.25%	0 0.0%	0.6
female	20 58.8%	9 26.4%		10 62.5%	5 31.25%	
Size						
≤ 2	14 41.2%	6 17.6%	0.1	5 31.25%	3 18.75%	0.5
>2 - ≤ 4	6 17.6%	3 8.8%		6 37.5%	2 12.5%	
>4	1 2.9%	4 11.7%		0 0	0 0	
Site						
Right	10 29.4%	3 8.8%	**0.04***	6 37.5%	2 12.5%	0.8
Left	7 20.6%	2 5.9%		3 18.75%	2 12.5%	
Bilateral	4 11.8%	8 23.5%		2 12.5%	1 6.25%	
Focality						
Unifocal	17 50%	5 14.7%	**0.02***	9 56.25%	2 12.5%	0.2
Multifocal	4 11.8%	8 23.5%		2 12.5%	3 18.75%	
Lympho-ascular Invasion						
Negative	18 53%	7 20.6%	0.05	10 62.5%	1 6.25%	
Positive	3 8.8%	6 17.6%		1 6.25%	4 25%	**0.01***
Perineural Invasion						
Negative	19 55.9%	9 26.5%	0.1	11 68.75%	3 18.75%	0.08
Positive	2 5.8%	4 11.8%		0 0.0%	2 12.5%	
Nodal Status						
Negative	18 52.9%	8 23.5%	0.2	11 68.75%	1 6.25%	**0.003***
Positive	3 8.8%	5 14.7%		0 0.0%	4 25%	
ETE						
Negative	20 58.8%	9 26.5%	0.05	11 68.75%	1 6.25%	**0.003***
Positive	1 2.9%	4 11.7%		0 0.0%	4 25%	
Tumor Necrosis						
Negative	19 55.9%	7 20.6%	**0.03***	11 68.75%	3 18.75%	0.08
Positive	2 5.8%	6 17.6%		0 0.0%	2 12.5%	
Grade						
Low	19 55.9%	7 20.6%	**0.03***	11 68.75%	3 18.75%	0.08
High	2 5.8%	6 17.6%		0 0.0%	2 12.5%	
Stage						
pT1	12 (35.3%)	4 (11.8%)		5 (31.25%)	0 (0)	
pT2	6 (17.6%)	3 (8.8%)	0.13	6 (37.5%)	1 (6.25%)	
pT3	3 (8.8%)	4 (11.8%)		0 (0)	4 (25%)	**0.007***
pT4	0 (0)	2 (5.8%)		0 (0)	0 (0)	

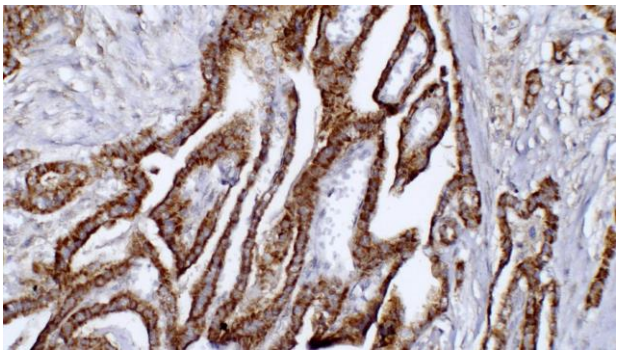

Figure 1: High cytoplasmic expression of SIX1 in classic papillary thyroid carcinoma (IHC, X400).

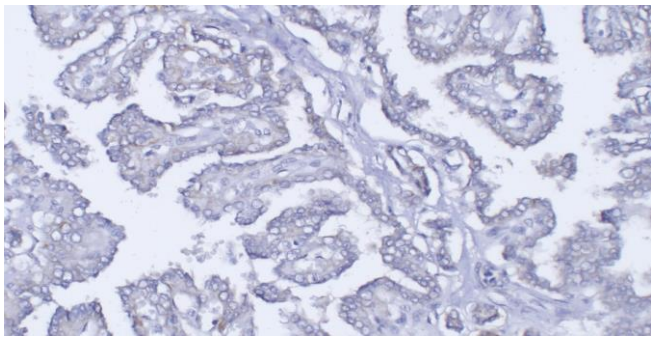

Figure 2: low cytoplasmic SIX1 expression in classic papillary thyroid carcinoma (IHC, X200).

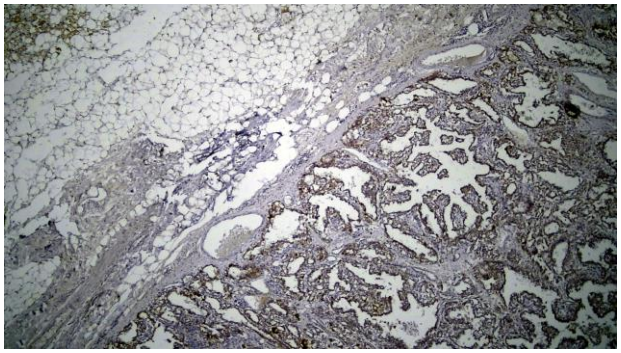

Figure 3: High cytoplasmic expression of SIX1 in lymph node infiltrated by classic papillary thyroid carcinoma (IHC, X40).

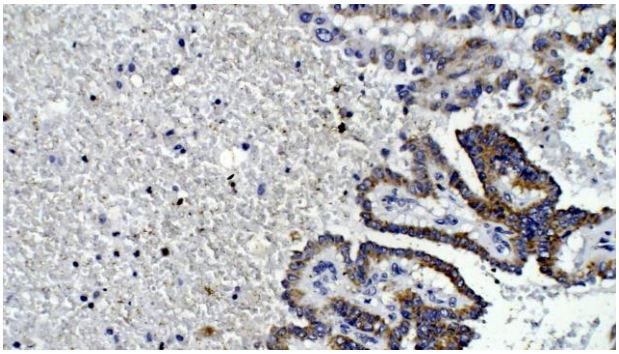

Figure 4: tumor necrosis in classic papillary thyroid carcinoma showing high cytoplasmic expression of SIX1 (IHC, X200).

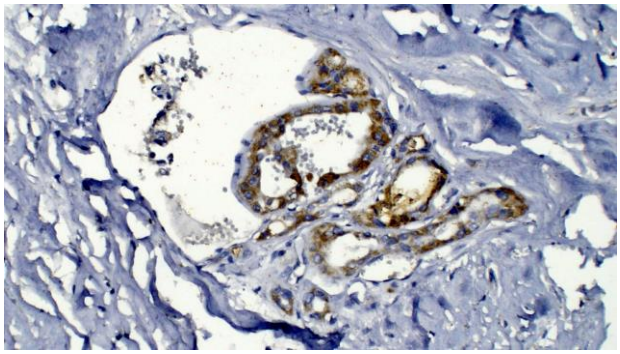

Figure 5: Vascular invasion in classic papillary thyroid carcinoma showing high cytoplasmic expression of SIX1 (IHC, X200).

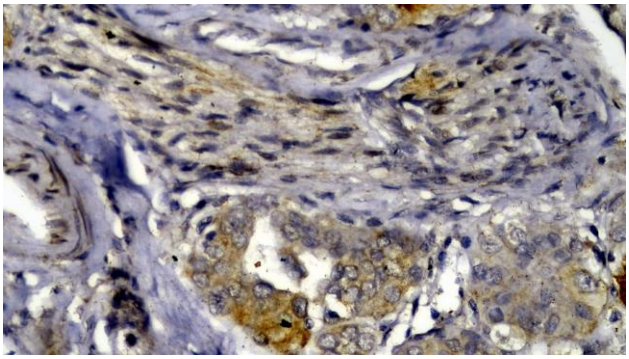

Figure 6: Perineural invasion in follicular variant of papillary thyroid carcinoma showing high cytoplasmic expression of SIX1 (IHC, X400).

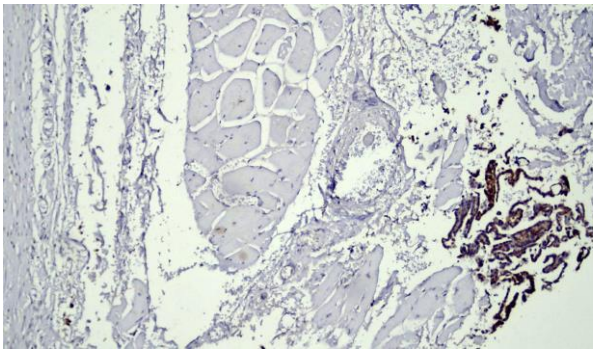

Figure 7: extra thyroidal extension in classic papillary thyroid carcinoma showing high cytoplasmic expression of SIX1 (IHC, X100).

Discussion

In our study, we have 50 cases of PTC where the mean age of our patients was 42.02 years ± SD 14.6, this was in a line with some previous studies performed by these studies [16.17.18]. On the other hand, other studies reported older mean of age 72.4± 6.24 years [19.20]. While other studies reported younger mean of age 13.8 years, ranging from 6 to 18 years [21.22].

Concerning patients' sex, 12% of patients were males and 88% were females. This was in accordance with previous studies where there is apparent female predominance[23.18]. However, it was in discordance with these studies [24.25.17], who reported less female predominance, 75% females and 25% of cases were males. While this study [26] reported that, male percentage more than the females, 56.7% and 43.3% respectively. This difference may be attributed to method of cases selection among different studies.

According to SIX1 expression, in our study expression of SIX1 was only cytoplasmic, this was in line with these studies [27.11]. While there were other studies reported that SIX1 expression could be nuclear and/or cyto-plasmic [12].

Regarding SIX1 expression, up to our knowledge, the current study reported 64% exhibited -ve /low cytoplasmic SIX1 expression, whereas 36% revealed high marker expression, that was in accordance with the following study [28], who reported that, pleuropulmonary blastoma had 26-50% showed SIX1 high expression. On the other hand, several other studies revealed a higher positivity from (52 to 63%) of PTC cases [12.13.11].

Regarding LNM, a statistically significant association was detected between SIX1 expression and lymph node status (P= 0.002), where 75% of cases positive for LNM showed high expression, while 25% of cases with positive for LNM showed low expression, this was in line with other studies [12.11], who reported that LNM in PTC was significantly associated with high SIX1 expression with (P = 0.043) and (P = 0.001) respectively and the following study[19], reported that LNM in head and neck squamous cell carcinoma was also significantly associated with high SIX1 expression (P = 0.0297). That was in discordance with [29], in this study there was no association between SIX1 expression and LNM in pancreatic cancer.

Concerning ETE, a statistically significant association was also detected between SIX1 expression and ETE (P= 0.001), where 88.9% of cases positive for ETE showed high expression, while 11.1% of cases negative for ETE showed low expression, that was in line with this study [11], which reported (p = 0.002), with positive rate 75.5%.

A statistically significant association was found between SIX1 expression and tumor pT

stage (P= 0.01), this was in accordance with these studies [27.30], who mentioned that, there was direct association between SIX1 expression in ovarian breast and liver cancers and advanced tumor stage in their study, and with those studies [12.11], who reported PTC staging was significantly associated with high SIX1 expression with (P = 0.015) and (P = 0.001) respectively. However, SIX1 expression was negatively associated with tumor (T) stage in endometrial carcinoma [24].
Little is known concerning SIX1 expression in primary PTC cases and the corresponding metastatic sites, either regional lymph nodes or distant metastasis. For more research about this issue, we evaluated SIX1 expression in 10 pairs of primary PTC cases and their corresponding LNM, our study is the only one to research the comparison of SIX1 expression in the primary tumor and corresponding lymph node metastasis.

Our results came up with that SIX1 expression was frequently maintained during metastasis from primary tumors to their corresponding LNM with a concordance rate of 80%. Most of tumors (75%) with high SIX1 expression in primary tumors were also high in their corresponding lymph node. This finding suggests that SIX1 bearing PTC cells have more ability to metastasize to lymph node and this is in line with [12], who reported that SIX1 increases the ability of PTC cells to invasion.

In our study, we found that high SIX1 is significantly expressed in CPTC with bilaterality, multifocality, high grade features, and tumor necrosis. While in FVPTC, high SIX1 is significantly expressed with LNM, advanced tumor stage, LVI and ETE. The most important finding in FVPTC group, that is not found in CPTC, is the strike association between SIX1 expression and LNM, where all cases with LNM showed high SIX1 expression. It was suggested that patients with LNM had a higher mortality, and the incomplete surgical excision was an important reason for the increased mortality in PTC patients of early stage [31]. Our data suggests that SIX1 may be beneficial in predicting patients who in need to perform prophylactic central lymph node dissection in cN0 FVPTC.

To conclude, our study demonstrated that SIX1 might be used as malignancy hallmarks in PTC. High SIX1 expression was found correlated poor prognostic factors in PTC. Moreover, SIX1 expression may predict different prognostic factors in CPTC and FVPTC. However, we recommend further studies on larger scale of CPTC and FVPTC to validate our results.

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