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PTEN loss is associated with follicular variant of Middle Eastern papillary thyroid carcinoma

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Background: PTEN gene at chromosomes 10q23.3 is a tumour suppressor gene that is inactivated in many types of human cancers. The known mechanisms of PTEN inactivation are rendered to mutation, epigenetic silencing by aberrant methylation or gene deletion. Although PTEN role has been documented in many cancers, PTEN alteration in papillary thyroid carcinoma (PTC) has not been fully elucidated. The aim of this study is to comprehensively investigate PTEN alterations in a large cohort of Middle Eastern papillary thyroid cancer by immunohistochemistry and fluorescent *in situ* hybridisation (FISH).

Methods: PTEN protein expression was analysed by immunohistochemistry in a tissue microarray (TMA) format in a large cohort of more than 1000 patients with papillary thyroid cancer. Copy number changes in PTEN were analysed by FISH and data were correlated with clinicopathological parameters along with survival analysis.

Results: PTEN inactivation reflected by complete absence of staining was seen in 24.5% of PTC samples, whereas PTEN deletion was seen only in 4.8% of the tested samples by FISH. No association was seen between PTEN loss of protein expression and PTEN gene deletion. However, interestingly, PTEN loss of expression was significantly associated with the follicular variant subset of papillary thyroid cancer.

Conclusion: Our study confirmed that PTEN might have a role in pathogenesis in a subset of PTC. PTEN loss of protein expression is a more common event in follicular variant of papillary thyroid cancer. Lack of association between PTEN loss of protein expression and PTEN gene deletion might indicate that gene deletion may not be the sole cause for PTEN loss of expression and these results might raise the possibility of other mechanism such as promoter methylation-mediated gene silencing to be responsible for PTEN inactivation.

Papillary thyroid carcinoma (PTC) is the most common malignant thyroid tumour representing 80–90% of all thyroid malignancies (Davies and Welch, 2006; Antonelli *et al*, 2011; Nixon *et al*, 2012). There is a worldwide increase in the incidence of PTC (Hussain *et al*, 2013; Ito *et al*, 2013). Increase in the incidence of thyroid cancers could be partially attributed to the increased use of PET scans and Doppler thereby leading to early detection (Yun *et al*, 2010; Nilsson *et al*, 2011). Other contributing factors include radiation exposures, obesity, diet and lifestyle (Pellegriti *et al*, 2013). In Saudi Arabia, thyroid cancer is the most common malignancy among females after breast cancer and accounts for 7.4% of overall cancers and 10.6% of all female malignant neoplasm (Bazarbashi, 2010; Hussain *et al*, 2013).

Follicular variant of papillary thyroid carcinoma (FVPTC) is the second most common histological subtype among PTCs.

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Although prognosis of FVPTC is not different from conventional phenotype (Zidan *et al*, 2003), there is increasing evidence that suggest FVPTC is composed of distinct biological entities (Yu *et al*, 2013). Follicular variant of PTC have higher frequency of allelic loss of heterozygosity of tumour suppressor genes as compared with conventional PTCs (Hunt *et al*, 2004).

The phosphoinositol 3-kinase (PI3K) AKT signal transduction pathways contributes to tumourigenesis and survival, and is activated in many cancer types including thyroid cancer (Chang *et al*, 2003; Campos *et al*, 2014; Danielsen *et al*, 2014; Manfredi *et al*, 2014; Porta *et al*, 2014). One of the main regulator of PI3K/AKT pathway is the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) located at 10q23.3 which is a tumour suppressor gene that acts by negatively regulating the PI3K/AKT pathway (Carracedo and Pandolfi, 2008; Georgescu, 2010).

The mechanism of PTEN inactivation can be attributed to several mechanisms such as point mutation (Goschzik *et al*, 2014; Sun *et al*, 2014), deletion (Cordes *et al*, 2013; Lotan *et al*, 2015), promoter hypermethylation (Hou *et al*, 2008; Piras *et al*, 2014) and post-translational modification (Yang *et al*, 2013). Among post-translational modifications, phosphorylation is the dominant mechanism of PTEN modification (Vazquez *et al*, 2000; Torres aand Pulido, 2001). The molecular basis of PTEN inactivation in thyroid carcinogenesis is still not fully elucidated. Several reported mechanism of PTEN inactivation in thyroid cancer varies from deletion (Dahia *et al*, 1997), mutation (Halachmi *et al*, 1998) to gene methylation (Alvarez-Nunez *et al*, 2006). However, the reported incidence of mutations in PTEN genes are low in papillary thyroid tumours (Liu *et al*, 2008; Sozopoulos *et al*, 2010). Deletion as well as loss of heterozygosity of the PTEN gene is suggested to have a greater role in thyroid carcinogenesis (Dahia *et al*, 1997; Halachmi *et al*, 1998). Aberrant methylation causing silencing of the PTEN gene is also been known to enhance the signalling of PI3K/AKT pathway, contributing to the progression of thyroid tumours (Hou *et al*, 2008). PTEN is hence considered an important tumour suppressor in thyroid carcinogenesis whose deficiency could lead to thyroid tumourigenesis (Bruni *et al*, 2000) and progression (Hou *et al*, 2008).

Previous studies performed on thyroid cancer for the role of PTEN have been contradictory. One reason for this discordance can be attributed to small sample size. Therefore, we took the advantage of our existing large scale thyroid cancer TMA that includes more than 1000 PTC with follow-up data to investigate the incidence of PTEN alterations in Saudi PTC. PTEN loss was evaluated by IHC in TMA format and fluorescence *in situ* hybridisation (FISH) was used to assess the overall frequency of PTEN copy number change in PTC. PTEN alteration was studied for correlation with clinicopathological parameters and also for any impact on clinical outcome.

MATERIAL AND METHODS

Patient selection and tissue microarray construction. One thousand and forty patients with PTC diagnosed between 1988 and 2011 were selected from King Faisal Specialist Hospital and Research Centre. All PTCs were analysed in a tissue microarray (TMA) format. Clinical and histopathological data were available for all the patients. Long-term follow-up data were available for most of the patients. TMAs were constructed with two-fold redundancy from formalin-fixed, paraffin-embedded PTC specimens as described previously (Kononen *et al*, 1998). Tumour regions were mapped by a pathologist for coring. The TMA was constructed with 0.6 mm diameter cores spaced 0.8 mm apart using a tissue microarrayer (Semi automated Arrayer, CM1 Mirlacher, Neuenburg, Germany). The TMA block was cut into 5 μm sections, adhered to a slide by an adhesive tape-transfer method (Instrumedics, Hackensack, NJ, USA) and UV cross-linked. The Institutional Review Board of King Faisal Specialist Hospital and Research Centre approved the study under Project RAC# 206008 on PTC archival clinical samples.

Evaluation of histological subtypes. Histological subtypes were classified according to World Health Organization (2004) classification. Papillary carcinoma was diagnosed based on characteristic features such as nuclear enlargement, nuclear crowding, ground glass appearance, nuclear grooving and nuclear pseudoinclusions. Tall cell variant was diagnosed when tumour composed predominantly of tumour cells with height three times their width, nuclear features of papillary carcinoma and plentiful oxyphilic cytoplasm. Follicular variant of PTC was diagnosed when tumour composed entirely of follicles of different shapes and size with virtually no papillae formation and cells lining the follicles possessing nuclear features of papillary carcinoma.

Immunohistochemistry. Standard protocol was followed for IHC staining. For antigen retrieval, Dako (Dako Denmark A/S, Glostrup, Denmark) Target Retrieval Solution pH 9.0 (Catalog number S2368) was used, and the slides were microwaved at 750 W for 5 min and then at 250 W for 20 min. Tissue microarray sections were stained with primary antibody PTEN (clone 6H2.1, Dako, 1:50 dilution, pH9) and p27 (clone 57, Invitrogen (Invitrogen Corporation, Carlsbad, CA, USA), 1:400 dilution, pH9). The Dako Envision Plus System kit was used as the secondary detection system with 3, 3′-diaminobenzidine as chromogen. All slides were counterstained with haematoxylin, dehydrated, cleared and mounted. Negative controls included omission of the primary antibody. Normal tissues of different organ system were also included in the TMA to serve as control. Only fresh cut slides were stained simultaneously to minimise the influence of slide aging and maximise reproducibility of the experiment. For immunoscoring of PTEN, only intensity score was taken into consideration (0–no staining, 1–weak, 2–moderate and 3–strong). Cases showing $\geqslant 1+$ intensity score was considered as positive for PTEN expression. Score 0 was considered as loss of PTEN expression. For p27, tumour cells showing staining of any intensity of $\geqslant 1+$ in $\geqslant 50\%$ of tumour cell was considered as positive.

Immunohistochemical scoring was done by two pathologists (SB and SP), blinded to the clinicopathological characteristics. Discordant scores were reviewed together to achieve agreement.

PTEN fluorescent *in situ* hybridisation. For PTEN, dual-colour FISH on paraffin-embedded TMA was performed using commercially available DNA probes LSI PTEN/CEP 10; Vysis Inc (Abbott Laboratories, Abbott Park, IL, USA). The PTEN locus-specific probe located on cytoband 10q23 was labelled with Spectrum Orange and centromere of chromosome 10 probe region 10p11.1-q11.1 was labelled with Spectrum Green (LSI PTEN/CEP 10; Vysis Inc.). The PTEN genomic probe spans 368 kb and starts 166 kb from 5′ end of the gene and extends 98 kb past the 3′ end of the gene. Histologic TMA tissue sections of 5 μm thickness were deparaffinised with a series of xylene prior to immersion in 100% ethanol. Fluorescent *in situ* hybridisation was carried out according to the manufacturer's instructions.

The FISH analyses for PTEN were performed independently and without knowledge of the immunohistochemical result. The PTEN /CEP17 ratios were calculated as stated in the manufacturer's guidelines. A cell with two signals of green (centromere 10) and two signals of orange (PTEN) was considered as normal or a PTEN /CEP17 ratio of 1 was considered normal; a tumour cell with two green (centromere 10) and one orange signal (PTEN) with a ratio of 0.5 was considered a hemizygous deletion; a tumour cell with two green and total absence of orange signal (PTEN) and a ratio of 0 was considered a homozygous deletion.

Statistical analysis. The JMP 10.0 (SAS Institute Inc., Cary, NC, USA) software package was used for data analyses. We examined the association of PTEN alterations with clinicopathological parameters, biomarker expression and also performed survival analysis. Survival curves were generated using Kaplan–Meier method with significance evaluated using the Mantel-Cox log-rank test. Values of $P<0.05$ were considered statistically significant.

RESULTS

Clinicopathological features. The mean age of the patients at initial surgery was 40.4 years (range 6–92 years). Of the patients, 261 were (25.1%) males and 779 (74.9%) were females. The mean duration of follow-up was 76.5 months (range 0–280 months). A total of 791 (78.3%) tumours were classical papillary carcinomas; 153 (15.1%) were follicular variant of papillary thyroid carcinoma; and 66 (6.5%) were tall cell variant. Extrathyroidal extension was seen in 462 (52.9%) cases and American Joint Committee on Cancer staging was as follows: 693 (68.6%) stage I; 51 (5.1%) stage II; 84 (8.3%) stage III; and 182 (18.0%) stage IV.

Table 1. Correlation of PTEN-IHC with clinico-pathological parameters in PTC

	Total		PTEN expression		PTEN expression loss		
	No.	%	No.	%	No.	%	*P* value
No. of patients	992		749	75.5	243	24.5	
Age (years)							
⩽45	628	63.3	469	74.7	159	25.3	0.4275
>45	364	36.7	280	76.9	84	23.1	
Sex							
Female	744	75.0	571	76.8	173	23.2	0.1186
Male	248	25.0	178	71.8	70	28.2	
Extrathyroidal extension							
Absent	391	46.7	284	72.6	107	27.4	0.0337
Present	446	53.3	352	78.9	94	21.1	
pT							
pT1	257	26.7	184	71.6	73	28.4	0.2944
pT2	197	20.5	156	79.2	41	20.8	
pT3	410	42.7	313	76.3	97	23.7	
pT4	97	10.1	73	75.3	24	24.7	
pN							
pN0	377	40.9	271	71.9	106	28.1	0.0582
pN1	544	59.1	421	77.4	123	22.6	
Distant metastasis							
M0	937	94.7	708	75.6	229	24.4	0.9884
M1	53	5.3	40	75.5	13	24.5	
Stage							
I	658	68.3	493	74.9	165	25.1	0.6231
II	50	5.2	39	78.0	11	22.0	
III	80	8.3	56	70.0	24	30.0	
IV	176	18.3	136	77.3	40	22.7	
Histology type							
Follicular variant	144	15.0	101	70.1	43	29.9	0.0039
Papillary-classical	757	78.6	569	75.2	188	24.8	
Tall-cell variant	62	6.4	56	90.3	6	9.7	
Tumour focality							
Multifocal	462	49.8	356	77.1	106	22.9	0.3869
Unifocal	465	50.2	347	74.6	118	25.4	
p27							
Above 50%	86	8.9	73	84.9	13	15.1	0.0272
Below = 50%	880	91.1	657	74.7	223	25.3	
Disease-free survival							
5 years				77.4		77.3	0.2763

Abbreviations: IHC = immunochemistry; PTC = papillary thyroid carcinoma.

PTEN expression and its correlation with clinicopathological parameters. Immunohistochemical analysis of PTEN expression was interpretable in 992 PTC spots, and the incidence of PTEN loss of expression in our cohort was found to be 24.5% (243 of 992 spots). Loss of PTEN expression was more frequently detected in the follicular variant (29.9%) compared with classical and tall cell variant of papillary carcinoma (24.8% and 9.7%, respectively; $P=0.0039$). Loss of PTEN expression correlated significantly with absence of extrathyroidal extension ($P=0.0337$) (Table 1).

In addition, the loss of PTEN expression showed a significant association with p27 loss on IHC ($P=0.0272$). However, PTEN expression was not associated with age, gender, lymphovascular invasion and AJCC stage. There was no difference in survival between patients showing PTEN loss and those tumours expressing the protein ($P=0.2763$) (Figure 1).

PTEN deletion on FISH and its correlation with clinicopathological parameters. PTEN deletion by FISH was interpretable in 916 PTC spots, and the incidence of PTEN deletion in our cohort was only in 4.8% (44 of 916) of cases. PTEN deletion was significantly associated with old aged patients (above 45years) at the time of diagnosis ($P=0.0253$). No association was seen with any other clinical and pathological parameters (Table 2). We also could not find any statistical correlation between PTEN FISH deletion and protein loss by IHC. PTEN FISH deletion was not associated with any significant survival difference ($P=0.9063$) (Figures 2 and 3).

Follicular variant of papillary thyroid cancer and its clinicopathological parameters. When we compared FVPTC with the more common classical papillary subtype of PTC, we found that FVPTC was significantly associated with absent extrathyroidal extension ($P<0.0001$), smaller size ($P=0.0142$),

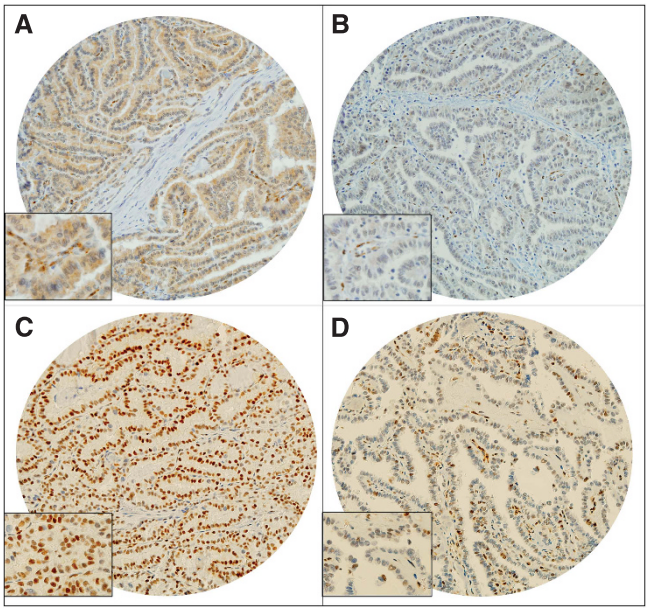

Figure 1. Immunohistochemical analysis of PTEN and p27 in papillary thyroid carcinoma. Tissue microarray spots showing expression of (**A**) PTEN and (**C**) p27. In contrast, different TMA spots showing loss of expression of (**B**) PTEN and (**D**) reduced expression of p27. Magnification (×20) on Olympus BX-51 microscope (Olympus America, Center Valley, PA, USA) with inset showing ×40 magnifications of the same TMA spot.

Table 2. Correlation of PTEN FISH with clinico-pathological parameters in PTC

	Total		PTEN Deleted		PTEN Normal/ Gain		
	No.	%	No.	%	No.	%	*P* value
No. of patients	916		44	4.8	872	95.2	
Age (years)							
⩽45	585	63.9	21	3.6	564	96.4	0.0253
>45	331	36.1	23	6.9	308	93.1	
Sex							
Female	687	75.0	29	4.2	658	95.8	0.1671
Male	229	25.0	15	6.6	214	93.4	
Extrathyroidal extension							
Absent	360	46.9	12	3.3	348	96.7	0.1209
Present	407	53.1	23	5.6	384	94.4	
pT							
pT1	235	26.6	10	4.3	225	95.7	0.8318
pT2	191	21.6	11	5.8	180	94.2	
pT3	372	42.1	18	4.8	354	95.2	
pT4	86	9.7	3	3.5	83	96.5	
pN							
pN0	348	41.2	18	5.2	330	94.8	0.2145
pN1	496	58.8	17	3.4	479	96.6	
Distant metastasis							
M0	868	95.0	41	4.7	827	95.3	0.5965
M1	46	5.0	3	6.5	43	93.5	
Stage							
I	609	68.7	25	4.1	584	95.9	0.2271
II	46	5.2	1	2.2	45	97.8	
III	77	8.7	7	9.1	70	90.9	
IV	155	17.5	9	5.8	146	94.2	
Histology type							
Follicular variant	133	15.0	8	6.0	125	94.0	0.3504
Papillary-classical	693	78.2	30	4.3	663	95.7	
Tall-cell variant	60	6.8	5	8.3	55	91.7	
Tumour focality							
Multifocal	429	50.1	23	5.4	406	94.6	0.5316
Unifocal	428	49.9	19	4.4	409	95.6	
p27							
Above 50%	86	9.7	5	5.8	84	94.2	0.5974
Below = 50%	798	90.3	36	4.5	762	95.5	
PTEN IHC							
High (1–3)	679	77.0	31	4.6	648	95.4	0.8315
Low (0)	203	23.0	10	4.9	193	95.1	
Disease-free survival							
5 years				80.6		77.6	0.9063

Abbreviations: IHC = immunochemistry; PTC = papillary thyroid carcinoma.

stage ($P = 0.0014$) and absence of regional lymph node metastasis ($P < 0.0001$). Strikingly, there was a significant positive association of FVPTC with distant metastasis ($P = 0.0026$) (Table 3).

DISCUSSION

Well-differentiated papillary thyroid carcinoma (PTC) accounts for about 90% of all thyroid cancers. Although the majority of these tumours tend to behave as indolent lesions, a small percentage of PTCs are highly aggressive and result in disseminated systemic spread to distant sites (Pellegriti *et al*, 2013). In our attempt to define molecular markers that might help to identify either aggressive behaviour or certain types of PTC, we screened the frequency of PTEN alteration in a large cohort of more than thousand PTC in TMA format given that the inactivation can occur through a variety of mechanisms.

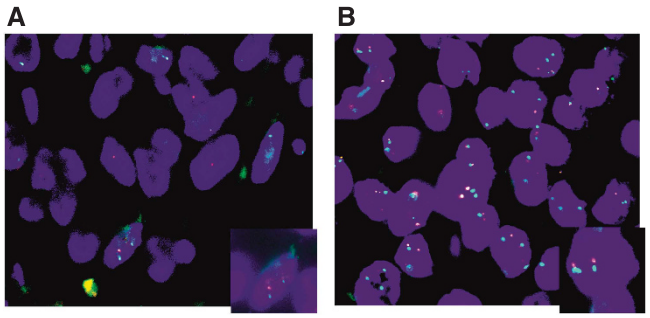

Figure 2. (**A**) Papillary thyroid carcinoma TMA spot showing deletion of PTEN gene (single red PTEN 2 signals and two green signals represent the reference gene of two centromeres of chromosome 10). Inset single cell. (**B**) Normal thyroid tissue spot showing no deletion (two red PTEN signals and two green centromeric signals). Inset single cell.

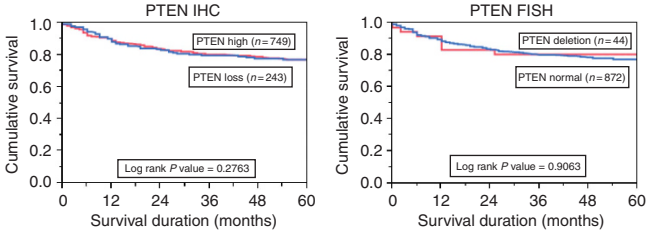

Figure 3. Kaplan–Meier survival curves. No significant survival difference was seen between patients showing PTEN protein expression loss and those expressing PTEN protein (**A**). Similarly, no significant survival difference was seen between patients showing PTEN deletion and those without deletion (**B**).

PTEN inactivation reflected by complete absence of PTEN by IHC was seen in 24.5% of our PTC cohort. Previous studies have reported differences in incidence of PTEN in different ethnic group which range from 29.7% (Duman *et al*, 2014) in Caucasian to 52.6% in Asian population (Min *et al*, 2013). Our results were similar to the Caucasian population as compared with other ethnicities.

PTEN inactivation in our patient cohort was not correlated with aggressive clinical parameters like nodal metastasis and extrathyroid extension reported by others (Min *et al*, 2013). However, PTEN loss of expression in our cohort was found to be significantly associated with FVPTC. Although FVPTC tend to behave clinically similar to classical PTC, several studies have hypothesised that FVPTC may reflect aggressive parameters like distant metastasis, extrathyroid extension, bilateral lesions and vascular invasion at the time of diagnosis (Hagag *et al*, 2006). Our study has also shown association between FVPTC and distant metastasis when compared with the classical subtype. Therefore, inactivation of PTEN in this particular subtype of PTC could probably have several clinical implications as other studies have revealed the diagnostic and clinical challenges associated with FVPTC as its molecular features are shared by both papillary thyroid cancers and follicular neoplasms (Salajegheh *et al*, 2008; D *et al*, 2014).

In our study, PTEN expression loss is found to be significantly correlated with loss of expression of cell cycle inhibitor p27, which might indicate that PTEN may have an important role in cell cycle regulation in PTC. These data are in concordance with another study that hypothesised PTEN suppressor gene is involved in upregulation of cell cycle inhibitor p27 (Bruni *et al*, 2000).

Table 3. Correlation of FVPTC with classical papillary subtype in PTC

	Total		Follicular Variant		Pap Classical Variant		
	No.	%	No.	%	No.	%	*P* value
No. of patients	944		153	16.2	791	83.8	
Age (years)							
⩽45	612	64.8	99	16.0	514	84.0	0.8259
>45	332	35.2	55	16.6	277	83.4	
Sex							
Female	709	75.1	118	16.6	591	83.4	0.5251
Male	235	24.9	35	14.9	200	85.1	
Extrathyroidal extension							
Absent	377	47.9	92	24.4	285	75.6	<0.0001
Present	410	52.1	35	8.5	375	91.5	
pT							
pT1	260	28.4	41	15.8	219	84.2	0.0142
pT2	194	21.2	45	23.2	149	76.8	
pT3	371	40.5	48	12.9	323	87.1	
pT4	91	9.9	11	12.1	80	87.9	
pN							
pN0	361	41.3	95	26.3	266	73.7	<0.0001
pN1	512	58.7	44	8.6	468	91.4	
Distant metastasis							
M0	894	94.9	136	15.2	758	84.8	0.0026
M1	48	5.1	16	33.3	32	66.7	
Stage							
I	639	69.7	99	15.5	540	84.5	0.0014
II	47	5.1	18	38.3	29	61.7	
III	74	8.1	12	16.2	62	83.8	
IV	157	17.1	19	12.1	138	87.9	
Tumour focality							
Multifocal	436	49.6	64	14.7	372	85.3	0.1303
Unifocal	444	50.4	82	18.5	362	81.5	

Abbreviations: FVPTC = follicular variant of papillary thyroid carcinoma; PTC = papillary thyroid carcinoma.

There are several mechanisms known for PTEN inactivation including gene deletion (Dahia *et al*, 1997), mutation (Steck *et al*, 1997) and promoter methylation (Whang *et al*, 1998). To test whether PTEN deletion might act as an important mechanism of PTEN inactivation, we have analysed PTEN deletion by FISH. Only 4.8% of our cohort showed PTEN gene deletion. No association seen between PTEN expression loss by IHC and PTEN deletion by FISH might indicate that PTEN gene deletion may not probably be the main mechanism for PTEN inactivation in Middle Eastern PTC. Even though PTEN promoter methylation leading to inactivation was not assessed in this study, lack of association between PTEN gene deletion and protein expression loss might strengthen the hypothesis that promoter methylation could be one of the main mechanism of PTEN inactivation. Our findings might pave the road for more studies to analyse the role of promoter methylation in PTEN inactivation in PTC.

In conclusion, PTEN loss is a common event in a subset of Middle Eastern PTC and gene deletion accounts for minority of PTEN protein expression loss. PTEN-mediated thyroid carcinogenesis have a greater role in FVPTC subtype. Lack of association between PTEN loss of protein expression and PTEN gene deletion might strengthen the hypothesis that PTEN inactivation in PTC is due to another mechanism such as promoter methylation rather than genomic alteration. Understanding the genetic landscape of these thyroid tumour subtypes will aid in accurately diagnosing, targeting and managing potential targets that have vital roles in PTC. Therefore, more studies are needed to understand the role of PTEN in PTC and mechanism behind its inactivation in this commonly occurring malignancy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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