Al-Kufa University Journal for Biology / VOL.11 / NO.3 / Year: 2019
Print ISSN: 2073-8854 Online ISSN: 2311-6544

p53, PTEN Tumor Suppressor Gene Methylation in Thyroid Cancer

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Abstract :

Background and objective: DNA methylation serves as an alternative to the mutational inactivation due to epigenetic event that alters gene expression without changing its DNA sequence. Such alterations may silence putative DNA repair genes and tumor suppressor genes whose role is undisputed in malignant transformation. This study was performed to detect the frequency of p53 and PTEN methylation in different thyroid cancers.

Method: Using methylation specific PCR methods, DNA was extracted from 76 surgically resected thyroid tissues with primary cancer. The target (p53, PTEN tumor suppressor genes) DNA was amplified with the mutation-specific primers.

Results: p53 gene complete methylation and partial methylation were 13.2%, and 17.1% respectively, whereas PTEN complete methylation and partial methylation were 18.4% and 23.7% respectively. Complete p53 methylation was significantly frequent among Anaplastic thyroid cancer (ATC) cases (50.0%) compared with other thyroid cancer, followed by Follicular thyroid cancer (FTC) (33.3%). Papillary carcinoma cases were more likely to be unmethylated for p53 gene. Despite the trend of negatively associated PTEN complete methylation with any of the studied clinicopathological parameters, however, merging partial with complete methylation gave a significant association of PTEN methylation with FTC and, inversely, there was low link with Papillary thyroid cancer (PTC).

Conclusion: Combined silencing of PTEN and p53 faithfully reproduces the development of ATC, and may provide a compelling rationale for a future target chemotherapy. p53 methylation appears low among early staged (T1, T2 and N0) tumors may indicate that p53 silencing is a hallmark of advanced tumors and so has bad ominous sign. PTEN methylation is also significantly associated with follicular carcinoma whereas, inversely, low in papillary carcinoma.

Keyword : *p53, PTEN Tumor, DNA methylation, Gene Methylation*

Introduction

Despite a relatively its low prevalence, thyroid cancer accounts for a disproportionate number of cancer-related deaths. Like other cancers, thyroid carcinoma initiation and advancement occur through gradual accumulation of numerous genetic modulation. Attentions have focused on p53 and phosphatase andtensin homolog (PTEN)

inactivation, through deletion, mutation or gene methylation, which has been found to be associated with advanced cancer type and stage [1;2]. A notice at a later stage disease, progressive loss of differentiation in high grade and advanced thyroid carcinomas are evolved by inactivation of p53 and PTEN genes [3;4;5; 6;7;8; 9].

The field of epigenetics refers to the genomic changes that alter gene expression without changing the DNA sequence itself. Such alterations may cause silencing of several putative DNA repair genes and tumor suppressor genes, whose role is undisputed during malignant transformation. DNA methylation that can serve as an alternative to mutational inactivation is a going to be a well-known epigenetic event in cancer [10]. Therapy researches on inactivated genes include transfection with wild-type gene enable to increase the therapeutic effect of radioiodine through regulating the expression of the Sodium/Iodide Symporter (NIS) [11, 12]. The current study might provide an insight into methylation signature of p53 and PTEN tumor suppressor gene in thyroid carcinoma in Duhok-Iraq.

METHODS

Patient selection and histologic evaluation: The study was conducted during a period extended from April, 2016 to February, 2017 in the General Laboratory and Research Center, Duhok-Iraq. Methylation was performed on 76 formalin fixed/ paraffin embedded thyroid cancer tissue collected from archives of the Histopathology Unit of General Central Laboratory and Vin Private Laboratory in Duhok-Iraq. No patient had received chemotherapy or radiation therapy prior to surgery. Clinical information about the patient's age, sex, tumor size and lymph nodes was obtained from the available histopathological reports and from patient's files in Azadi Teaching Hospital and Vin Private Hospital. Three mm-thick tissue sections were taken. Hematoxylin and Eosin (H&E) stained sections were re-examined by the pathologist for definite confirmation. Cases diagnosed histopathologically as anaplastic carcinoma were further subjected to immunohistochemistry (IHC) to confirm the diagnosis.

After definite diagnosis, cases were grouped into lymphocyte rich thyroiditis associated tumors and those without inflammation.

Tumor staging was based on the pathology report as well as the microscopic examination of the primary tumor, thyroid capsule, adjacent tissue and any associated structure if available (T). Sections from all the lymph nodes (N) were examined microscopically, this in addition to the clinical data concerning extrathyroid radiologic assessment for distant metastasis (M). Pathologic staging was given according to the WHO pathological (TNM) staging system [13].

Immunohistochemistry was carried out to confirm the diagnosis of anaplastic carcinoma and equivocal papillary and follicular carcinomas, using the fully automated immunostainers (Dako Denmark, Link48 and automated Ventana Benchmark) according to the instructions supplied by the manufacturer's (Ventana) and as described previously by some researchers [14,15,16]. Initially, a basic panel of antibodies (Vimentin, pankeratin and S-100 protein) was applied. Subsequently, an additional panel of antibodies (CK7, CK20, CK19, HBME, CD56, TG and TTF1) was used. For MTC, calcitonin, CD56, NSE and chromogranin were added [15;16].

Molecular Biology: Tumor DNA was extracted by proteinase K digestion (6 ng/mL at 55C for 18 to 24 hours under continuous stirring) and a QIAamp DNA FFPE Tissue Kit (QAIamp DNA FFPE Tissue Handbook, 2012). Then DNA was measured

by Nano Drop and Gel electrophoresis. The methylation specific PCR was used to analyze p53 and PTEN methylation in 76 thyroid cancer samples. The molecular findings were monitored using electrophoresis on 1 % agarose gel containing Ethidium Bromide dye and directly visualized under UV illumination. Data analysis was achieved according to the instructions provided by the kits.

Statistical analysis.

Statistical analysis were performed by Statistical Package for Social Sciences 24 (SPSS 24; IBM Corp; USA). Pearson Chi-square and Fishers' exact test. The predictors of p53 and PTEN methylation and un-methylation positivity was examined in univariate analysis of variance. The P-value of less than 0.05 was considered statistically significant difference.

RESULTS

The mean patient's age was 41.04 years (ranged, 19-72; median, 40) with 1:2 male to female ratio. Pathologically, 63 (82.9%) cases were papillary carcinoma, 9 (11.8%) follicular carcinoma, 4 (5.3%) anaplastic carcinoma. Concerning the stage, 30 (39.5%) cases were at T1 followed by 27 (35.5%) at T4, 12 (15.8%) at T3 and 7 (9.2%) at T2. Forty three (56.6 %) cases were at N0 and 33 (43.4%) were at N1. Metastasis couldn't be assessed in 75 (98.7 %) cases (MX). Only one case was reported to have M1 that is positive distant metastasis.

PTEN complete methylation, partial methylation and unmethylation were 18.4%, 23.7% and 57.9% respectively. Whereas p53 gene complete methylation, partial methylation and unmethylation in thyroid cancer were 13.2%, 17.1% and 69.7% respectively (Figure 1).

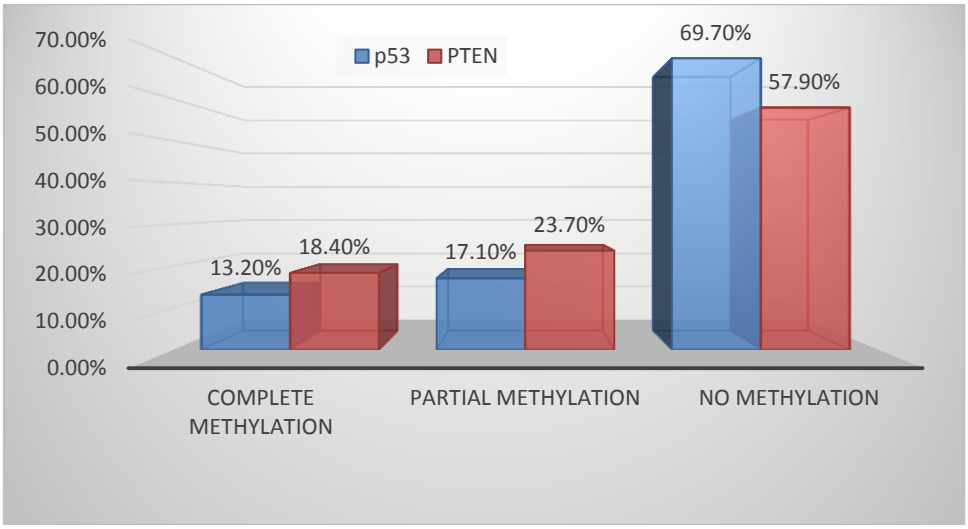

Figure 1. Methylation statuses of p53 and PTEN genes in thyroid cancer cases

P53 methylation

Depicted gels of p53 methylation are illustrated in Figure 2.

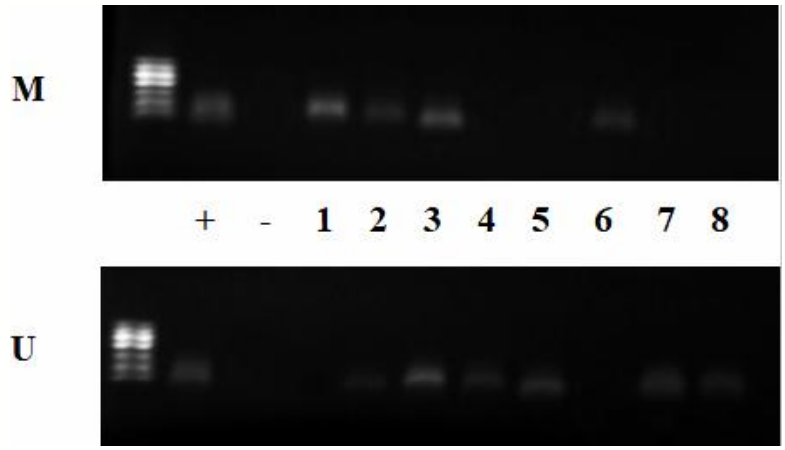

Figure 2. Methylation-specific PCR of the p53 promoter region in tumor samples.

Plus symbol universally methylated positive control DNA, minus symbol universally negative control DNA without DNA. The presence of a visible PCR product in the lane U indicates the presence of unmethylated p53 genes, the presence of product in the lane M indicates presence of methylated p53 genes. Sample no. 1, 6 have complete methylated promoter region of p53 gene and sample no. 2, 3 has partial methylated promoter region of p53 gene and sample no. 4, 5, 7, 8 unmethylated.

A shown in Table 1, complete methylation of p53 gene was significantly high among ATC cases (50.0%), followed by FTC (33.3%). Papillary carcinoma cases were more likely to harbor unmethylated p53 gene (p= 0.004). This methylation was significantly low among low tumor stages, T1/T2 and negative lymph node metastasis (p< 0.001 and 0.002) respectively. Descending frequency of ***Phi*** refers to the likelihood methylation of p53 gene among ATC with its decline among T1/T2 and N0 and (*Phi*: 0.552, *Phi*: 0.407 and *Phi*: 0.421) respectively. No statistical differences could be detected between the mentioned methylation and any of sex, thyroiditis-associated cancers and papillary variants.

Combining partial and complete p53 methylation together, emphasized the significantly high association of p53 methylation with ATC in addition to its significant low frequency among PTC and FTC (p= 0.002), T1 and T2 (p< 0.001) and nodal negativity N0 (p= 0.002).

Table 1: p53 methylation and clinicopathological parameters

Clinical Features	p53 gene methylation					
	Complete	Partially	Negative	*p*-Value	Combined (complete + partial)	*p*-Value
Thyroid cancer ATC PTC FTC	2 (50.0) 5 (7.9) 3 (33.3)	1 (25.0) 9 (14.3) 3 (33.3)	1 (25.0) 49 (77.8) 3 (33.3)	0.004** *Phi*: 0.421	3 (75.0) 14 (22.2) 6 (66.7)	0.002** *Phi*: 0.387
Sex Male Female	2 (9.1) 8 (14.8)	1 (4.5) 12 (22.2)	19 (86.4) 34 (63.0)	0.120** *Phi*: 0.243	3 (13.6) 20 (37.0)	0.044** *Phi*: -0.231
Tumor (T) status T1 T2 T3 T4	0 (0.0) 0 (0.0) 2 (16.7) 8 (29.6)	2 (6.7) 0 (0.0) 4 (33.3) 7 (25.9)	28 (93.3) 7 (100.0) 6 (50.0) 12 (44.4)	<0.001** *Phi*: 0.552	2 (6.7) 0 (0.0) 6 (50.0) 15 (55.6)	<0.001** *Phi*: 0.530
Lymph node (N) N0 N1	3 (7.0) 7 (21.2)	3 (7.0) 10 (30.3)	37 (86.0) 16 (48.5)	0.002* *Phi*: 0.407	6 (14.0) 17 (51.5)	0.002* *Phi*:-0.405
Metastasis (M) MX M1	9 (12.0) 1 (100.0)	13 (17.3) 0 (0.0)	53 (70.7) 0 (0.0)	0.132** *Phi*: 0.297	22 (29.3) 1 (100.0)	0.303** *Phi*: -0.175
Associated Thyroiditis Negative Hashimoto Lymphocytic	7 (12.1) 2 (18.2) 1 (14.3)	12 (20.7) 0 (0.0) 1 (14.3)	39 (67.2) 9 (81.8) 5 (71.4)	0.527** *Phi*: 0.197	19 (32.8) 2 (18.2) 2 (28.6)	0.762** *Phi*: 0.111
PTC variant Conventional Microscopic Follicular Columnar	5 (10.6) 0 (0.0) 0 (0.0) 0 (0.0)	5 (10.6) 1 (10.0) 2 (50.0) 1 (50.0)	37 (78.7) 9 (90.0) 2 (50.0) 1 (50.0)	0.208** *Phi*: 0.366	10 (21.3) 1 (10.0) 2 (50.0) 1 (50.0)	0.241** *Phi*: 0.238
*Pearson Chi-square and **Fishers' exact tests were performed for statistical analyses.						
N0: No spread to nearby lymph nodes; N1: Positive spread to nearby lymph nodes;						

MX: Metastasis cannot be assessed; M1: Positive distant metastasis.

PTEN methylation

Depicted gels of PTEN methylation is illustrated in Figure 3.

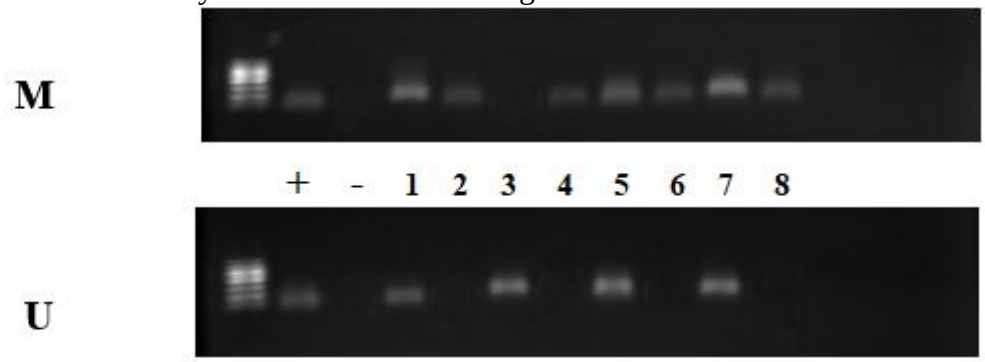

Figure 3. Methylation-specific PCR of the PTEN promoter region in tumor samples. (+) universally methylated positive control DNA, (-) universally negative control without DNA. The presence of a visible PCR product in the lane U indicates the presence of unmethylated PTEN genes, the presence of product in the lane M indicates presence of methylated p53 genes. Sample no. 1, 5, 7 have partial methylated promoter region of PTEN gene and sample no. 2, 4, 6, 8 has complete methylated promoter region of PTEN gene and sample no. 3, unmethylated.

Despite the trend of negative PTEN methylation toward T1, N0, associated thyroiditis and papillary carcinoma, no significant association of PTEN gene complete methylation with any of the studied clinicopathological parameters. Merging partial with complete methylation, gave a significant association of PTEN methylation with FTC and, inversely, low link with PTC (p= 0.028). As well, although statistically not significant, a trend of negative PTEN methylation was found toward T1 and N0 and PTC variants with a relatively favorable prognosis "conventional, follicular and microcarcinoma" (Table 2).

Table 2: PTEN gene methylation in regards to clinicopathological status of the cases (n= 76).

Clinical features	PTEN gene methylation					
	Complete	Partial	Negative	*p*-Value	Combined (complete + partial)	*p*-Value
Thyroid cancer ATC PTC FTC	1 (25.0) 10 (15.9) 3 (33.3)	1 (25.0) 13 (20.6) 4 (44.4)	2 (50.0) 40 (63.5) 2 (22.2)	0.104** *Phi*: 0.273	2 (50.0) 21 (33.3) 7 (77.8)	0.028** *Phi*: 0.297
Sex Male Female	4 (18.2) 10 (18.5)	4 (18.2) 14 (25.9)	14 (63.6) 30 (55.6)	0.751* *Phi*: 0.087	7 (31.8) 23 (42.6)	0.383* *Phi*: -1.00
Tumor (T) status T1 T2 T3 T4	3 (10.0) 2 (28.6) 4 (33.3) 5 (18.5)	5 (16.7) 1 (14.3) 2 (16.7) 10 (37.0)	22 (73.3) 4 (57.1) 6 (50.0) 12 (44.4)	0.202** *Phi*: 0.332	8 (26.7) 2 (28.6) 5 (41.7) 15 (55.6)	0.342** *Phi*: 0.297
Lymph node (N) N0 N1	7 (16.3) 7 (21.2)	7 (16.3) 11 (33.3)	29 (67.4) 15 (45.5)	0.129* *Phi*: 0.232	13 (30.2) 17 (51.5)	0.060* *Phi*: -0.216
Metastasis (M) MX M1	14 (18.7) 0 (0.0)	18 (24.0) 0 (0.0)	43 (57.3) 1 (100.0)	1.000** *Phi*: 0.098	30 (40.0) 0 (0.0)	1.000** *Phi*: 0.093
Associated Thyroiditis Negative Hashimoto Lymphocytic	12 (20.7) 2 (18.2) 0 (0.0)	12 (20.7) 5 (45.5) 1 (14.3)	34 (58.6) 4 (36.4) 6 (85.7)	0.241** *Phi*: 0.280	22 (37.9) 7 (63.6) 1 (14.3)	0.122** *Phi*: 0.246
PTC variant Conventional Microscopic Follicular Columnar	7 (14.9) 0 (0.0) 2 (50.0) 1 (50.0)	12 (25.5) 1 (10.0) 0 (0.0) 0 (0.0)	28 (59.6) 9 (90.0) 2 (50.0) 1 (50.0)	0.145** *Phi*: 0.393	18 (38.3) 1 (10.0) 1 (25.0) 1 (50.0)	0.327** *Phi*: 0.230
*Pearson Chi-square and **Fishers' exact tests were performed for statistical analyses.						

Coexistence of both P53 and PTEN genes methylation in the studied cases:

As illustrated in Table 3, absence of both PTEN and p53 methylation was statistically more pronounced among T1 status, negative nodal metastasis, thyroiditis-carcinoma coexistence (p= 0.001, < 0.001, 0.02) respectively. As well, this double lack of methylation was more dominant among papillary type carcinoma. In contrast, coexistence of both gene methylation was more prominent among follicular type carcinoma (p= 0.001).

Table 3. Coexistence of p53 and PTEN methylation in the studied cases (n= 76).

Database	Methylation status				
	P53+/ PTEN+	P53+/ PTEN-	P53-/ PTEN+	P53-/ PTEN-	*P*-Value
Sex N (%) Male Female	 1 (4.5) 11 (20.4)	 6 (27.3) 12 (22.2)	 2 (9.1) 9 (16.7)	 13 (59.1) 22 (40.7)	0.231**
Tumor N (%) T1 T2 T3 T4	 1 (3.3) 0 (0.0) 3 (25.0) 8 (29.6)	 7 (23.3) 2 (28.6) 2 (16.7) 7 (25.9)	 1 (3.3) 0 (0.0) 3 (25.0) 7 (25.9)	 21 (70.0) 5 (71.4) 4 (33.3) 5 (18.5)	0.001**
Lymph node N (%) N0 N1	 5 (11.6) 7 (21.2)	 8 (18.6) 10 (30.3)	 1 (2.3) 10 (30.3)	 29 (67.4) 6 (18.2)	<0.001*
Metastasis N (%) MX M1	 12 (16.0) 0 (0.0)	 18 (24.0) 0 (0.0)	 10 (13.3) 1 (100.0)	 35 (46.7) 0 (0.0)	0.145**
Thyroiditis N (%) Negative Positive	 11 (19.0) 1 (14.3)	 11 (19.0) 7 (63.6)	 8 (13.8) 3 (32.5)	 28 (48.3) 7 (89.6)	0.021**
PapillaryN (%) Conventional PTC Microcarcinoma PTC-follicular PTC Columnar ATC FTC	 3 (6.4) 0 (0.0) 1 (25.0) 1 (50.0) 1 (25.0) 6 (66.7)	 15 (31.9) 1 (10.0) 0 (0.0) 0 (0.0) 1 (25.0) 1 (11.1)	 7 (14.9) 1 (10.0) 1 (25.0) 0 (0.0) 2 (50.0) 0 (0.0)	 22 (46.8) 8 (80.0) 2 (50.0) 1 (50.0) 0 (0.0) 2 (22.2)	0.001**
Age (Mean/SD)	47.00 (7.9)	41.3 (14.3)	45.2 (16.8)	36.7 (11.8)	0.061**

*Pearson Chi-squared, **Fishers' exact, and *** Independent t-tests were performed for statistical analyses. +: Methylation, -: No methylation

Discussion

p53 Methylation

Inactivation of p53 has been identified as a serious tumor suppressor gene in a variety of other human cancers [17,18]. Surprisingly, little has been published in the literature about p53 methylation in thyroid malignancy. This methylation was identified in 30.3% of the tested thyroid cancers.

An interesting finding in the present study was the identification of p53 methylation in three (two complete and one partial) out of four anaplastic carcinoma which belongs to the most aggressive thyroid cancers. Although the tested number was too small, however p53 methylation in 75% of ATC and its low frequency among PTC and FTC (which belong to the low grade thyroid cancers) may reflect the association of p53 silencing with ominous thyroid cancers. Studies have investigated p53 mutation rather than methylation among thyroid cancers, mainly anaplastic carcinoma. The frequency of this mutation ranged from 55%-88%, which is rather too high among anaplastic thyroid carcinoma despite the generally small sample sizes (Table 3).

Another important finding was that p53 gene methylation was obviously low or nearly absent among early staged (T1 and T2) tumors and negative nodal metastasis (N0). Studies have shown that p53 inactivation, as a result of genetic alteration or epigenetic silencing, is frequent in advanced and aggressive thyroid cancer phenotypes [19, 20, 21, 22, 23]. In oncology prospect, our results are consistent with the findings of the effect of p53 methylation on the progress of different human cancers [24, 25].

Table (3): p53 methylation in thyroid cancer in the literature.

Study	p53 Alteration	Sample number	Country	Cancer type and frequency			
				Totally	PTC	FTC	ATC
Current, 2019	Methylation	76	Iraq	30.3	22.2	66.7	75.0
Liu *et al*, 2017[11]	Mutation	51	China	-	-	-	56.86
Landa *et al*, 2016[33]	Mutation	33	USA	-	-	-	73.0
Latteyer *et al*, 2016[34]	Mutation	39	Germany	-	-	-	60.0
Smallridge *et al*, 2009[21]	Mutation	22	USA	-	-	-	55.0
Quiros *et al*, 2005[9]	Mutation	8	USA	-	-	-	88.0

In two separate studies, Yamashita, *et al* have found that usually full methylation of the DNA promoter (rich in CpG bi- nucleotides) is crucial for complete gene deactivation, but although small fractions of unmethylated alleles might strongly induce gene expression [26, 27]. In primary tumor cells, super high methylation (SHM) represents complete methylation in the cancer cells, and SHM in those primary cancer tissues was actually presenting with low protein expression of the genes of interest. Their results concluded that SHM of p53 gene pathway might be functionally similar to p53 functioning defect. Although positive prognostic impact of p53 mutation is

controversial, some researchers have supported this relationship [28] while others were dissenters of this possibility [29]. The significant association between the p53 methylation and the ATC status that has been found in the current study, is supported by other studies, in a study conducted by Adrian *et al*, they analyzed the changes in the methylation profile of the CpG dinucleotides within the *TP53* gene promoter in -139 and -132, they revealed the presence of methylated cytosines in these places and the majority of the tested DNA samples in their study showed the presence of methylation of both alleles (complete methylation), while the methylation of a single allele (partial methylation) was seen in lower rate among the studied patients [30]. From those results, it can be postulated that a complete methylation (involving both alleles) can demonstrate higher predisposition toward malignant tumor development. As well, hypermethylation of the promoter region of the *TP53* tumor suppressor gene is usually related with impaired its activity, and may results in its suppression, which in turn leads to function loss.

PTEN Methylation

Experts in this field assumed that PTEN absence is associated with tumor progression [31, 2, 32]. In this study, PTEN methylation (complete and partial) was significantly associated with follicular carcinoma (77.8%). The second major finding was that anaplastic carcinoma (50%). Inversely, 66.7% of papillary carcinomas lacked PTEN methylation, although both tested follicular variant PTC showed PTEN methylation. Research issue with respect to PTEN, its deletion was reported in 28–83% of thyroid malignancies with PTEN silencing by aberrant promoter methylation in more than 80% of follicular carcinoma and 69% of anaplastic carcinoma (Table 4).

In an attempt to explore biomolecular markers that is assumed to help identifying the behavior of PTC, Beg *et al* in their large cohort of more than one thousand Saudi patients with PTC, reported that PTEN loss to be significantly associated with the follicular variant subset of papillary thyroid cancer, Also they found in their study the loss of expression of PTEN was significantly associated with follicular variant PTC. Although this variant tends to behave clinically similar to classical PTC but genetically similar to follicular carcinoma. This collaborates the concept of genetic connections between follicular carcinoma and follicular variant – papillary carcinoma [31]. Although statistically not significant, there was a trend of lacking PTEN methylation early tumor status (T1 and T2) and negative nodal metastasis (N0).

Table (4): PTEN methylation in thyroid cancer in the literature.

Study	PTEN Alteration	Sample number	Country	Cancer type and frequency			
				Totally	PTC	FTC	ATC
Current study, 2019	Methylation	76	Iraq	42.1	33.3	77.8	50.0
Beg *et al*, 2015[31]	Methylation	1000	Saudi Arabia	-	24.5	-	-
Smallridge *et al*, 2009[21]	Mutation	22	USA	-	-	-	12.0
Hou *et al*, 2008[8]	Methylation	143	USA	-	-	51.0	69
Alvarez-Nunez *et al*, 2006[2]	Methylation	98	Spain		45.7	83.3	-
Frisk *et al*, 2002[32]	Mutation	87	Sweden	-	-	-	28.6

Exclusively in this study, cases with combined lacking of PTEN and p53 methylation was statistically more pronounced among early staged cancers (T1 and N0) and in cases of associated thyroiditis. Such finding raises the argument that combined lacking of PTEN and p53 methylation is associated with better prognostic parameters. As well, double lack of methylation was more dominant among the papillary type carcinoma in contrast to the more prominent coexistence of both gene methylation with the follicular type carcinoma, a finding that may weigh the follicular carcinoma toward a less favorable outcome than PTC.

Conclusion

The methylation of p53 was identified in high frequency of ATC, low frequency among PTC and FTC with obviously low or nearly absent among early staged (T1, T2 and N0) tumors, may indicate that p53 silencing is a bad omen and reflects advanced tumors. PTEN methylation is significantly associated with follicular carcinoma. Notwithstanding the relatively limited sample, this work also offers valuable insights PTEN into anaplastic carcinoma. The combined lacking of PTEN and p53 methylation among early staged cancers (T1 and N0) and in cases of associated thyroiditis, can be taken as a good prognostic parameter.

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