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.
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 (Goschikz 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 and 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 H121, 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 immunoscopying of PTEN, only intensity score was taken into consideration (0–no staining, 1–weak, 2–moderate and 3–strong). Cases showing ≥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 ≥1+ in ≥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 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.

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 (\( P = 0.0276 \)). 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 45 years) 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 (\( P = 0.0142 \)), and absence of extrathyroidal extension (\( P = 0.0272 \)).

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

| Parameter                  | No. | %   | No. | %   | P value |
|----------------------------|-----|-----|-----|-----|---------|
| Age (years)                |     |     |     |     |         |
| < 45                       | 629 | 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 | 70.1 |43       | 29.9 | 0.0039  |
| 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 | 76.9 |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  | 75.5 |24       | 24.7 |         |
| pN                         |     |     |     |     |         |
| pN0                        | 377 | 40.9| 271 | 77.4 |106      | 28.1 | 0.0582  |
| pN1                        | 544 | 59.1| 421 | 72.6 |132      | 22.6 |         |
| Distant metastasis         |     |     |     |     |         |
| M0                         | 932 | 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  | 80.0 |11       | 20.0 |         |
| 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  | 6.6 | 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  | 89.6 |13       | 10.4 | 0.0272  |
| Below = 50%                | 880 | 91.1| 657 | 74.7 |223      | 25.3 |         |
| Disease-free survival      |     |     |     |     |         |
| 5 years                    | 774 | 77.3| 77.3| 27.63  |        |         |

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

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.
In our study, PTEN expression loss is found to be significantly correlated with distant metastasis (P = 0.0026) (Table 3). The significance of PTEN expression loss was also supported by Kaplan–Meier survival curves, showing a positive correlation with distant metastasis (Figure 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.

PTEN inactivation reflected by complete absence of PTEN by IHC was observed in 24.5% of our PTC cohort. Previous studies have reported differences in incidence of PTEN in different ethnic groups, ranging from 29.7% (Duman et al, 2014) in Caucasian to 52.6% in Asian population (Min et al, 2013). Our study also showed similar results, with no significant difference in PTEN expression between Caucasian and other ethnic populations.

PTEN inactivation in our patient cohort was not correlated with aggressive clinical parameters like nodal metastasis and extrathyroidal 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, extrathyroidal 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 2. Correlation of PTEN FISH with clinico-pathological parameters in PTC**

| Parameter                        | PTEN Deleted | PTEN Normal/Gain | P value |
|----------------------------------|--------------|------------------|---------|
| Total No. of patients            | 749          | 872              | 0.0253  |
| Age (years)                      |              |                  |         |
| ≤ 45                             | 583          | 44               | 4.8     | 95.2   | 0.0001  |
| > 45                             | 331          | 24               | 6.9     | 93.1   |         |
| Sex                              |              |                  |         |
| Female                           | 687          | 29               | 4.2     | 95.8   | 0.0167  |
| Male                             | 229          | 19               | 6.6     | 93.4   |         |
| Extrathyroidal extension         |              |                  |         |
| Absent                           | 430          | 12               | 3.3     | 94.4   | 0.1209  |
| Present                          | 360          | 23               | 5.6     | 94.6   |         |
| pT                               |              |                  |         |
| PT1                              | 236          | 10               | 4.3     | 95.7   | 0.8318  |
| PT2                              | 191          | 11               | 5.8     | 94.2   |         |
| PT3                              | 372          | 18               | 4.8     | 95.2   |         |
| PT4                              | 86           | 4                | 4.7     | 95.3   |         |
| pN                               |              |                  |         |
| pN0                              | 348          | 18               | 5.2     | 94.8   | 0.2145  |
| pN1                              | 496          | 17               | 3.4     | 96.6   |         |
| Distant metastasis               |              |                  |         |
| M0                               | 868          | 41               | 4.7     | 95.3   | 0.5965  |
| M1                               | 46           | 3                | 6.5     | 93.5   |         |
| Stage                            |              |                  |         |
| I                                | 609          | 25               | 4.1     | 94.0   | 0.2271  |
| II                               | 46           | 1                | 2.2     | 97.8   |         |
| III                              | 77           | 7                | 9.1     | 90.9   |         |
| IV                               | 155          | 9                | 5.8     | 94.2   |         |
| Histological type                |              |                  |         |
| Follicular variant               | 133          | 8                | 6.0     | 125    | 94.0    | 0.3504  |
| Papillary-classical              | 693          | 30               | 4.3     | 663    | 95.7    |         |
| Tall-cell variant                | 60           | 5                | 8.3     | 146    | 91.7    |         |
| Tumour focality                  |              |                  |         |
| Multifocal                       | 429          | 23               | 5.4     | 406    | 94.6    | 0.3316  |
| Unifocal                         | 428          | 19               | 4.4     | 409    | 95.6    |         |
| p27                              |              |                  |         |
| Above 50%                        | 86           | 5                | 5.8     | 84     | 94.2    | 0.5974  |
| Below 50%                        | 798          | 36               | 4.5     | 762    | 95.5    |         |
| PTEN IHC                         |              |                  |         |
| High (1–3)                       | 679          | 31               | 4.6     | 648    | 95.4    | 0.8315  |
| Low (0)                          | 203          | 10               | 4.9     | 193    | 95.1    |         |
| Disease-free survival            |              |                  |         |
| 5 years                          | 80.6         | 77.6             | 0.9063  |
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 between PTEN expression loss and PTEN gene deletion was observed 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 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 mechanisms of PTEN inactivation. Our findings might pave the road for more studies to analyse the role of promoter methylation in PTEN inactivation in PTC.

The authors declare no conflict of interest.

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PTEN loss in papillary thyroid carcinoma

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