The validity of Immunohistochemical Expression of PTEN protein in detecting PTEN gene deletion in Prostate cancer.

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الخلاصة

جين PTEN هو جين الكابتة للورم موجود فى كروموسوم 10q23,3 ذو خاصية مزدوجة حيث يزيل الفوسفات عن البروتينات والدهون حاملا لمماثل مجال tensin . بروتين PTEN يقوم بإرسال اشارة تنظيم انقسام الخلايا ويوجه الخلايا لدخول مسار موت الخلايا الطبيعى, فقدان هذا الجين يؤدى الى تحفيز بروتين AKt وهذا بدوره يرتبط مع انقسام الخلايا غير المنضبط.

في هذه الدراسة المقطعية، تم اخذ50 عينة من المقاطع النسيجية تنتمي إلى 50 مريضا مصابين بسرطان البروستاتا، لتقييم مرحلة Gleason ودرجة التمايز، وربطها بمستوى بروتينPSA في الدم ,قبل الجراحة والإعراض السريرية والنتائج المرضية الأخرى.

كافة الشرائح تعرض للدراسة بواسطة المعلمات المناعية النسيجية مع PTEN لتقييم التعبير عن بروتين PTEN والدراسة الجزيئية لجين PTEN في جميع الأنسجة باستخدام تقنية CISH

أظهرت النتائج أن 54.55٪ (06/11) من حالات حذف PTEN المتخالف كان تعبير البروتين PTEN فيها سلبيا، وكانت الحالات المتبقية 45.45٪ (05/11) ذات تعبير ضعيف لبروتين PTEN , في حين أن جميع حالات الحذف المتماثل لجين PTEN أظهرت تعبير سلبى لبروتين PTEN.لذلك تدعم دراستنا بقوة الأثر الهام لحذف الجين PTEN في سرطان البروستاتا على النشاط التكاثري للخلايا الخبيثة , وأيضا تؤيد استخدام طريقة المعلم المناعي النسيجي للتحري عن فعالية الجين في سرطان البروستاتا

Abstract

PTEN gene is a tumor suppressor gene located in 10q23,3 that encode dual-specificity protein and lipid phospatase with tensin homologe. PTEN signaling regulate cell division and direct cells to enter natural cell death pathway. loss of PTEN leads to over –activation of AKt, which in turn , is associated with uncontrolled cell proliferation. In this cross section study, we examined 50 paraffin-embedded blocks belongs to 50 patients with proved prostate cancer, All slides subjected to IHC PTEN Ab to assess the expression of PTEN protein , and to molecular study of PTEN gene in all tissues by using CISH technique. The results show that 54.55% (6/11) of the heterozygous PTEN deletion had negative PTEN
protein expression, and the remaining cases 45.45% (5/11) had weak –positive PTEN expression , while all the cases of homozygous deletion of PTEN gene show negative PTEN protein expression.

Introduction

prostatic cancer is still one of the major health problems all over the world ,and is one of the chief causes of cancer mortality in men. It forms a significant percentage of hidden malignancies existing with secondary metastasis [1] . prostate cancers exhibit a variable range

of clinical and behavioral forms ,ranging from slow-growing tumors of slight clinical significance to extremely aggressive metastatic and lethal recurrent disease.[1]

The PTEN gene , also known as MMAC1 or TEP1 which is a tumor suppressor gene located on chromosome 10q23,3 that encodes a dual-specificity protein and lipid phosphatase and tensin homolog , it targets the PIP3, converting it back to PIP2 has an important role in determinining the aggressive behavior of prostate cancer[2].

PTEN signaling regulates cell division and can also direct cells to enter a natural cell death pathway. As a regulator of PI3K signaling[3] , loss of PTEN leads to over –activation of AKt, which in turn , is associated with uncontrolled cell proliferation and decrease apoptosis.

Inactivation of PTEN is frequent in prostate cancer, specially the metastatic types , placing PTEN mutation among the most common genetic alterations reported in human prostate cancer[5].

CISH is a cytogenetic procedure that join the chromogenic signal detection method of immunohistochemistry (IHC) with in situ hybridization. CISH techniques used to detect the presence or absence of specific regions of DNA ,as perform in FISH technique[6] . Though, CISH is much more practical in diagnostic laboratories because it uses bright-field microscopes rather than the more expensive and complicated fluorescence microscopes used in FISH,CISH probes are labeled with biotin or digoxigenin.[6]

and can be detected by bright-field microscope after treatment steps have been used [6].

So the aim of the present study is to is to clarify the sensitivity of immunohistochemical expression of PTEN protein to the genomic state of PTEN gene in prostate cancer.

Materials and methods

Samples collection

In this cross sectional study, included 50 paraffin embedded prostate cancer specimens from the archive of department of pathology in Al-Sader teaching hospital and the private laboratories with reference guide to the patients files from the Middle Euphrates center for oncology.

50 sample are collected for those with total prostatectomy and the trucut biopsies that proved as prostate carcinoma, after we exclude all the specimens that lacking the clinical data, full investigations, or those with a controversy about their diagnosis. All the clinical data were collected from patient file : age, ultrasonographic reports, preoperative serum PSA. Sectioning of paraffin embedded blocks done by using the Leica rotary microtome in department of pathology/Al-Kufa Collage of Medicine.

Staining the slides

Before staining , the slides left in electric oven at 60°C to deparaffinizing them, for at least 30 minutes. The usual stain Hematoxylin and Eosin (H&E) is used to provide essential structural information about the specimens. After staining, the sections are covered with a glass coverslip. Interpretation of slides done by authors to grading them according to last updated Gleason score.

Immunohistochemistry

Detection (staining) system:

(Dako EnVision + system-HRP(AEC) code K4004)

PTEN Antibody Kit

Monoclonal Mouse Anti-Human PTEN Clone 6H2.1(Dako)Code M3627

This antibody is intended for laboratory use to identify qualitatively by light microscopy PTEN expressing cells in normal and neoplastic tissues using immunohistochemical (IHC) test methods.

The cellular staining pattern for anti-PTEN is cytoplasmic and/or nuclear.[7]

Staining procedure

Apply Peroxidase block, enough optimally diluted primary antibody or negative control reagent to cover specimen, apply enough peroxidase Labeled Polymer, apply enough of the ready-to-use AEC+ substrate-chromogen solution, hematoxylin counterstain, then Specimens mounted and coverslipped with an aqueous-based mounting medium such as Glycergel Mounting Medium (code C0563). (Staining Protocol modified from Dako EnVision+ System-HRP protocol)

Evaluation of staining results

PTEN expression can be evaluated subjectively by estimation of the staining index , which was stratified from (0-9) scores .

The staining index obtained by multiplication of the cytoplasmic staining intensity of malignant prostate cells (0-3) , and the semi quantitative proportion of immunopositive tumor cells (0-3)

Staining intensity of malignant cells stratified as follow: score 0 (for no staining),Score 1 (for mild staining),Score 2 (for moderate staining).Score 3 (for obvious staining).

Proportion of immunopositive tumor cells calculate as follow:

Score 1 (for < 10% of tumor cells),Score 2 (for 10-50% of tumor cells),

Score 3 (for > 50% of tumor cells).The specimen considered Negative : in scores 0&1, considered weak-positive : in score 2&3, moderately-_positive : if the score 4 or 5,and sever-positive if the score 6-9. [8]

The ZytoDot 2C CISH Implantation Kit protocol

Molecular study of PTEN gene in which, Dual-color CISH was carried out on tissue sections , to map the PTEN gene on chromosome 10q23.3 region , the 5 µm tissue sections were de-paraffinized , proteolyses by pepsin solution, denaturation and hybridization with PTEN probe in overnight incubation, post hybridization apply Anti-DIG/DNP-Mix,_HRP/AP-Polymer-Mix,_AP-Red Solution, Apply HRP-Green Solution, counterstain and dehydrate the slides(modified from ZytoVision ZytoDot of ZytoVision GmbH catalogue2015).

Then we counted chromogenic signals in 100 non-overlapping interphase nuclei for each sample , so PTEN gene deletion was defined in tumor nuclei that contain one or no 10q23.3 locus signal (one or no green signals) , images were acquired with bright- field microscope.

Interpretation of CISH results

The PTEN probe is labeled with digoxigenin (DIG), that results in permanent dark-green signals, the probe also labeled with dinitrophenyl (DNP) that results in permanent bright-red signals by using AP-Red solution. By this procedure we can detect 2 green and 2 red distinct dot –shaped signals with rounded edges in each nucleus in normal diploid cells, but in mitotic cells additional signals may be visible (ZytoDot 2C CISH Implementation Kit catalogue2015).

Complete absence of probe signals (green signals) in ≥ 60% of tumor nuclei of the tissue spot, considered as homozygous deletion of PTEN gene , with presence of one or two green signals in the remaining nuclei, while absence of one probe signal (green signal) in ≥ 60% of tumor nuclei of tissue spot, was defined as heterozygous deletion of PTEN gene.[8]

Results

The mean age of patients participating in the current study was 68.18±8.72 years and their ages ranged from 48- 87 years. The mean prostatic specific antigen (PSA) serum level of the study population (pre operative measure) was 31.57±39.45 ng/ml and a median of 31.57 ng/ml.

The range of PSA was (0.9 up to >100 ng/ml). The patients were classified into four groups according to the serum level of PSA as follows (table1).

Table 1: Classification of patients according to serum PSA level

PSA	N	%
≤4 nmol/ml	12	24.00
>4-≤10 nmol/ml	14	28.00
>10-≤20 nmol/ml	4	8.00
>20 nmol/ml	20	40.00
Total	50	100.00

According to Gleason score, patients were categorized into: 22 patients (44%) with <7 score, 8 patients (16%) with score of 7 and the rest of patients (40%) with score of >7 (table 2). Median Gleason score was 7 and the range was 3-9.

Table 2: Classification of patients according to Gleason score

Gleason score	N	%
<7	22	44.00
7	8	16.00
>7	20	40.00
Total	50	100.00

According to PTEN immunohistochemistry, the following results were obtained (table 3) and figure (1):

Twenty six patients (52 %) had negative score, fourteen patients (28 %) had weak positive score, six patients (12 %) had moderate positive score and four patients (8 %) had strong positive score.

Table (3): Categorization of patients according to immunohistochemical PTEN expression.

PTEN score	N	%
Negative	26	52.00
Weak positive	14	28.00
Moderate positive	6	12.00
Strong positive	4	8.00
Total	50	100.00

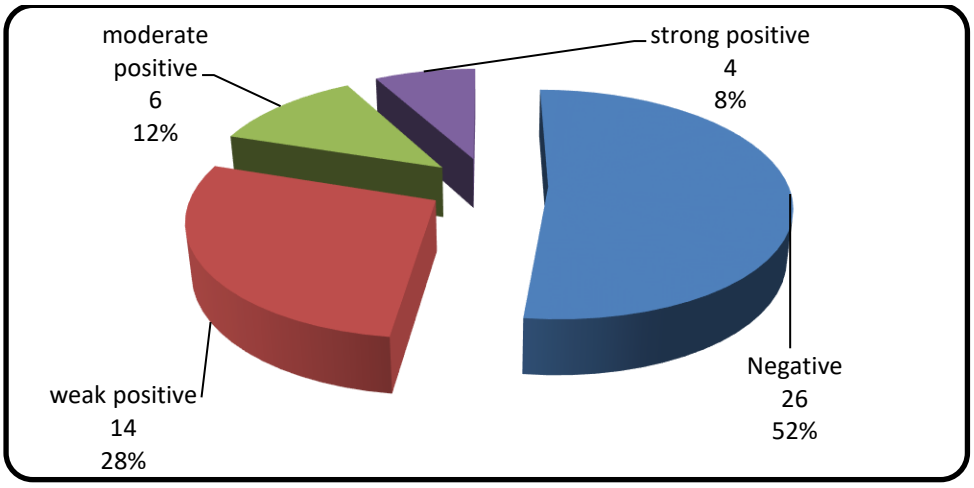


Figure (1): Pie chart showing the categorization of patients according to immunohistochemical PTEN expression.

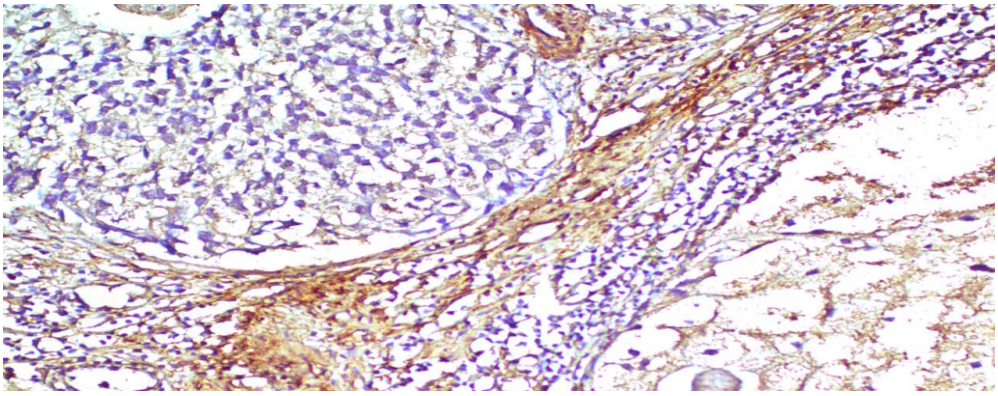


Figure 2. prostate cancer- PTEN protein expression score 0.(x40)

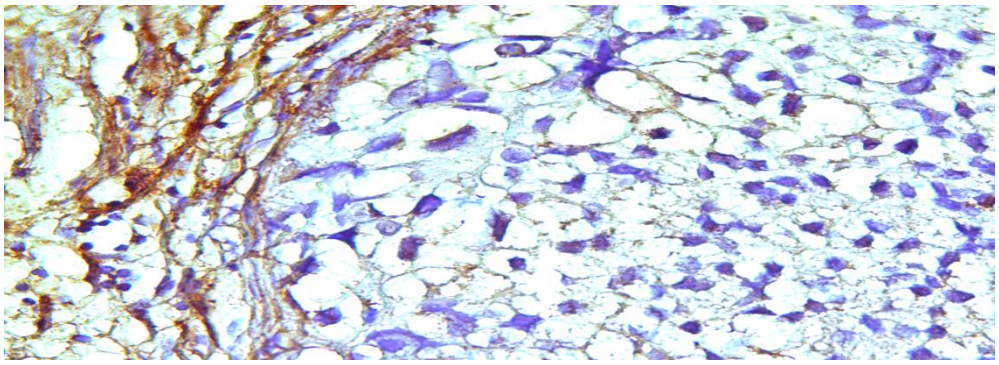


Figure 3. prostate cancer- PTEN protein expression score1. (X40)

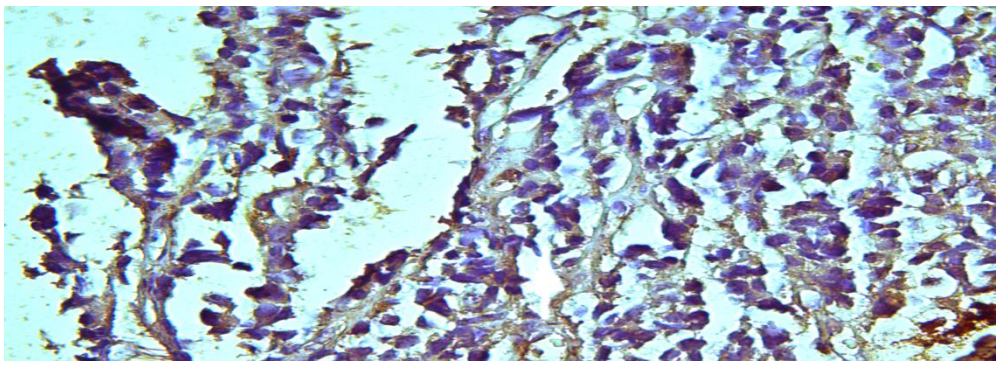


Figure 4. prostate cancer-PTEN protein expression score 2. (x40)

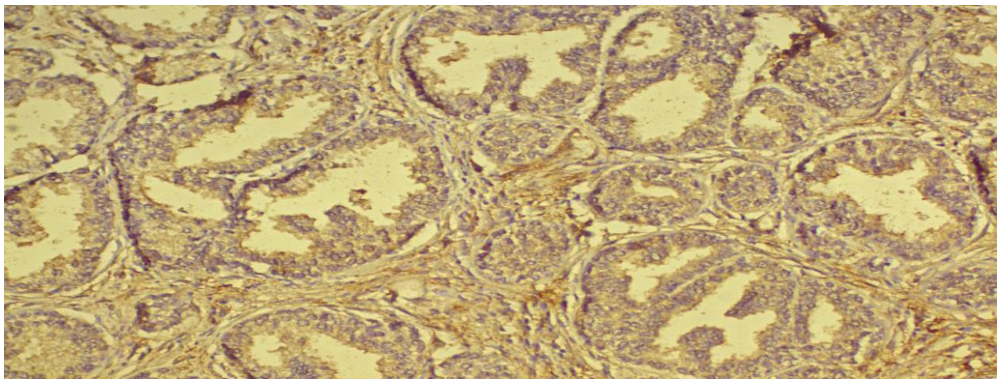


Figure 5. prostate cancer-PTEN protein expression score 3. (40)

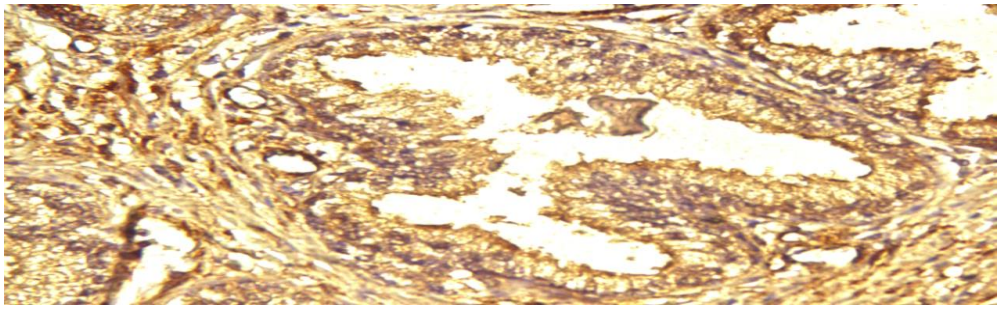


Figure 6.Benign prostatic hyperplasia -PTEN protein score 3(40)

According to CISH method, patients were found to have the following: deletion was present in 15 patients (30%): 4 of them showed homozygous genotype (8%) and 11 showed heterozygous genotype (22%). The rest of patients (70%) had no gene deletion (figure 7).

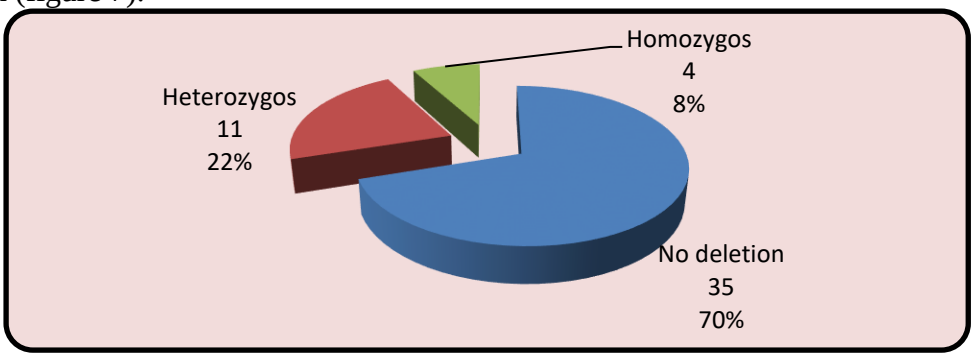


Figure 7.Pie chart showing the results of CISH procedure

Figure8.prostate cancer- CISH image show no PTEN deletion (401x400) oil immersion lens.

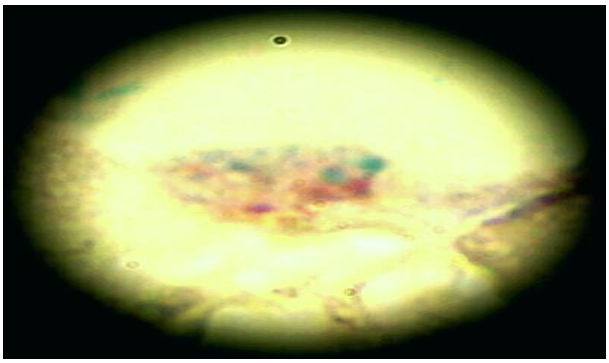
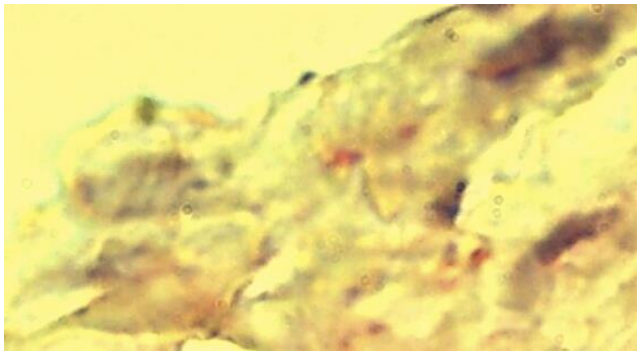


Figure 9. prostate cancer-CISH image show heterozygous deletion of PTEN gene

Figure 10.prostatec cancer. CISH image show homozygous deletion of PTEN 9565x306) oil immersion lens.



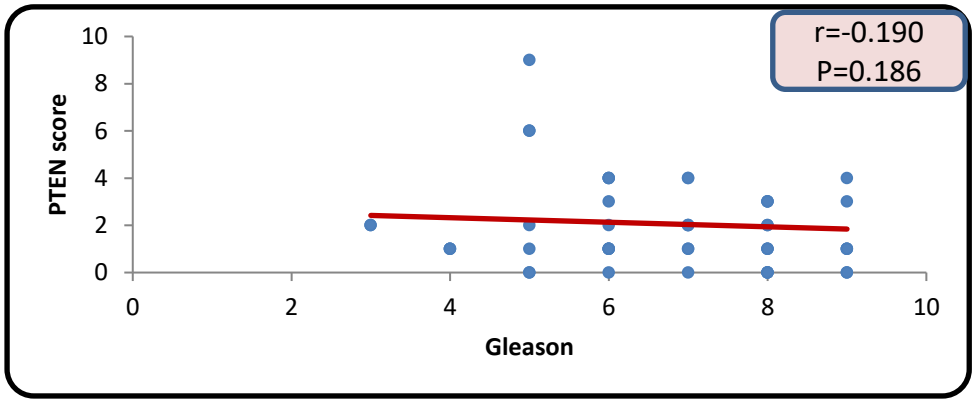
The association between Gleason score and PTEN immunohistochemical score is shown in table 4. P-value was not calculated because Ch-square test was not valid.

Table 4: Association between PTEN IHC expression and Gleason score.

	PTEN IHC score					
	Positive		Negative		Total	
Gleason*	N	%	N	%	N	%
≤7	11	50.00	11	50.00	22	100.00
7	5	62.50	3	37.50	8	100.00
≥7	8	40.00	12	60.00	20	100.00
Total	24	48.00	26	52.00	50	100.00

*Chi-Square is not valid because >20% of cells have expected count of <5.

The correlation between Gleason score and PTEN score was showed to be



insignificant using Kindals Tau b test (r=-0.190, P>0.05) (figure 11).

Figure 11: Correlation between Gleason score and PTEN IHC score

The association between CISH status of PTEN gene and PTEN IHC score is shown in table5. P-value was not calculated because more than 20% of the cells had expected count of less than 5.

Table 5: Association between PTEN IHC score and CISH results

	PTEN IHC									
	Negative		weak positive		moderate positive		strong positive		Total	
CISH	N	%	N	%	N	%	N	%	N	%
No deletion	16	45.71	9	25.71	6	17.14	4	11.43	35	100.00
Heterozygos	6	54.55	5	45.45	0	0.00	0	0.00	11	100.00
Homozygos	4	100.00	0	0.00	0	0.00	0	0.00	4	100.00
Total	26	52.00	14	28.00	6	12.00	4	8.00	50	100.00

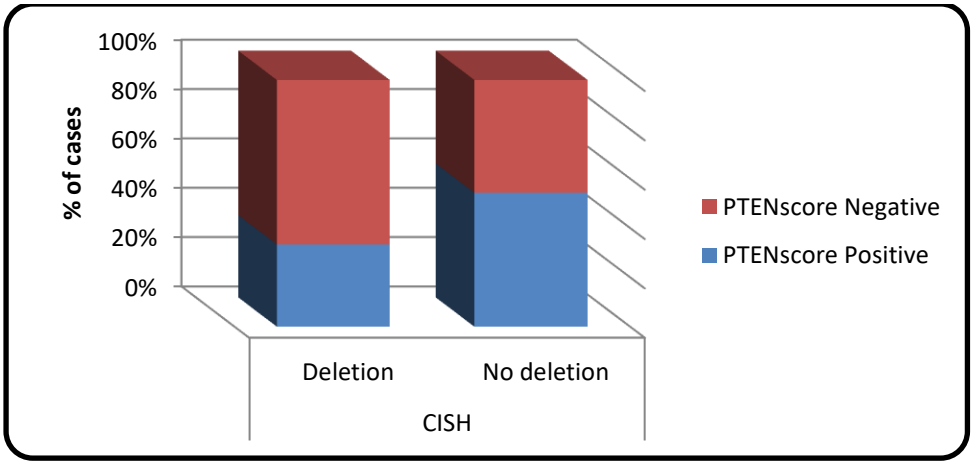


Figure 12. Concordance between CISH and IHC PTEN results.

The PTEN IHC method for detection of PTEN gene status had sensitivity 66.67% ,and 54.29% specificity.

Table (6): Sensitivity of PTEN IHC for detection of PTEN gene state.

PTEN IHC	CISH status	
	Deletion	No deletion
Negative	10	16
Positive	5	19
Sensitivity	66.67 %	
Specificity	54.29 %	

Discussion

The age distribution of our patients reflects the association of prostate cancer with old age patients. Were 40% of our patients are in 70-79 years intervals, and in seldom we find patient under 50 years(1 patient). 20 specimens (40%) have Gleason score >7, this permits to make good quality evaluation of aggressive cases in comparison to other cases (<7 &7 score cases) by molecular and immunohistochemical study of PTEN gene and protein respectively. In this study , there is high range of PSA level (0.9 - >100 ng/ml) in the 50 cases of prostate cancer patients serum prior to operation (prostactomy or Trucut biopsy), and by classifying patients serum level of PSA in 4 categories , there is high percentage of patients 40 %(20/50) have PSA serum level > 20 ng/ml. this display the relative association between PSA level and prostate cancer.

In immunohistochemical study of slides stained with PTEN antibody, we obtained 26 slides (52%) have negative score (score 0+1) and 14 slides (28%) have weak-positive staining (score 2+3) as this combination depended by Ole J. Halvorsen et al (7) to get more valuable analysis of data , so that in 52% of cases there is no PTEN protein expression , in other ward no tumor suppression to prostate cancer ,and full activity of PI3K/Akt pathway towards the activating the cell cycle ,cell proliferation and prevent apoptosis of malignant cells, so we expected that 52% of cases have aggressive behavior , and 28% of cases have low PTEN protein expression ,that is not enough to suppress malignant cells proliferation.

In molecular study of PTEN gene status we used CISH technique as an alternative procedure to FISH, first because CISH is much more practical in diagnostic laboratories since it uses bright-field microscopes rather than the more expensive and complicated fluorescence microscopes used in FISH, second reason is that we can get a permanent images to the slides, permits to further evaluation of them by authors. Our results show that 15 cases (30%) have PTEN gene deletion ,from that 11 cases (22%) have heterozygous deletion of PTEN gene (deletion of one allele of gene) e.i; we found one green signal and two red signals,

in ≥60% of tumor cell nuclei, and 4 cases (8%) have homozygous

deletion of PTEN gene (deletion of both alleles of gene) e.i; we found two red signals only (no green signal) in ≥60 % of malignant cell nuclei.

The association between PTEN gene status and PTEN protein expression

The results show that 54.55% (6/11) of the heterozygous PTEN deletion had negative PTEN protein expression, and the remaining cases 45.45% (5/11) had weak –positive PTEN expression , while all the cases of homozygous deletion of PTEN gene , there is negative PTEN protein expression , e.i ; there is no PTEN protein expression or weak expression in all cases of PTEN deletion, so the immunohistochemical assay for PTEN protein is highly sensitive to state of PTEN gene in prostate cancer cells (in case of PTEN gene deletion).
But in non-deletion state , we had 45.71% (16/35) had negative protein expression, and 25.71% (9/35) had weak-positive score, this means that there are a significant number of prostate cancer cases had negative or weak expression of PTEN protein in spite that they had non-deleted PTEN gene , as we see that 61.53% (16/26) of the negative PTEN score in IHC had no-deletion of PTEN gene.

Lotan TL et al also found that PTEN immunohistochemistry is highly sensitive for detection of PTEN gene loss , that detect 80% of cases with loss by FISH [10] ,Yosshimoto M et al also found similar result[11], Verhagen PC et al found 66% (10/15) of cases with PTEN gene deletion showed PTEN protein loss (11). In the same way Lotan TL et al found that 45% of prostate tumors with PTEN protein loss did not show PTEN gene deletion in FISH [10].

The immunohistochemical method for detection of genomic state of PTEN had 66.67% sensitivity , and 54.29% specificity.

This result display that CISH technique may fail to detect some cases of prostate cancer with PTEN inactivation (loss of function), that strongly argues for the depends on the immunohistochemical method for detection of PTEN activity in prostate cancer cells.

Conclusion

The immunohistochemical PTEN protein scoring on prostate tumors specimens can give us highly informative information about the state of PTEN gene state in these tumors**.** And using the CISH enable to detect PTEN gene deletion in accurate scheme as occur in FISH technique. And it is more practical in diagnostic laboratories.

Recommendation

all these results recommended to use the IHC test for PTEN protein expression in the setting of prostate cancer because it can identify the additional cases lacking PTEN protein , which is the important factor in determine the aggressiveness and proliferating capacity of tumor.

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