بسم الله الرحمن الرحيم

Sudan University of Science and Technology
College of Graduate Studies

Immunohistochemical Detection of Cyclin d1 in Prostate Tumors among Sudanese Patients

الكشف المناعي النسيجي الكيميائي عن سايكلين دي 1 في اورام البروستاتا لدي المرضى السودانيين

A Dissertation Submitted in Partial Fulfillment for the Requirement of M.Sc. Degree in Medical Laboratory Science (Histopathology and Cytology)

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Dedication

To my father

Who taught me how challenging life could be

To my mother

God bless you and keep you in my life

To my

Brother and sisters

To my dear

Aunt Zinab may her soul rest in peace

Acknowledgments

If I’m in form of thanking, well praising Almighty Allah for giving me strength to carry on this research in a proper way

I owed deep gratitude to histopathology and cytology department in faculty of medical laboratory sciences, Sudan University of Science and Technology.

I am in debated to Dr. Abu Elgasim Abass Awad Elkareem for his supervision and support to crafting this research in correct way.

My sincere thanks to Miss Nehal and Omdurman Teaching Hospital for the cooperation.

This is great opportunity to express my respect to Mr. Abubakr Mahboob for his guide and useful information during the whole way.

I address my word of thanks to all my friends and colleagues for all support and effort.

Abstract

This is a hospital based analytical case control study; it was conducted at the Omdurman Teaching Hospital during the period from July 2017 to November 2017. The study was aimed to detect the expression of Cyclin d1 in prostate tumors in Sudanese patients.

A total of thirty formalin fixed paraffin blocks of previously diagnosed prostatic adenocarcinoma were included in the study. The grades of study samples selected were as follow: 6 (15%) samples were well differentiated prostate cancer, 9 (22.5%) samples were moderately differentiated prostate cancer and 15 (37.5%) samples were poorly differentiated prostate cancer. In addition ten formalin fixed blocks of prostatic hyperplasia were included 10 (25%).

A three µm thickness section was cut by rotary microtome from each block and stained by immunohistochemical method (avidin-biotin technique) for Cyclin d1detection. The data obtained was analyzed using SPSS computer program version 16.

Patients age ranged between 27 and 90 years with mean age of 69 years, most of the patients 38 (95%) were more than 50 years and the remaining 2(5%) were less or equal 50 years.

Positive expression of Cyclin d1 was detected in (29/30) of malignant tumor samples, while (1/30) malignant tumor sample showed negative expression and (10/10) of benign tumor samples were negative expression for Cyclin d1. This result showed significant association between expression of cyclin d1 and malignant tumor samples (P. value 0.000).

The positive expression of Cyclin d1 on well differentiated tumor was 6 (20%), moderately differentiated 9 (30%) and on poorly differentiated was 14 (46.7). Negative expression on poorly differentiated was 1 (3.3%) (P. value 0.596).

The study concludes that the positive expression of Cyclin d1 is associated with malignant prostate tumors more than benign tumors, with no association with tumor grade.

المستخلص

أجريت هذه الدراسة المستشفوية التحليلية والحالة الضابطة في مستشفى أم درمان التعليمي خلال الفتره من يوليو 2017 الى نوفمبر 2017 هدفت الدراسة للكشف عن دور سايكلين د1 في اورام البروستاتا لدى المرضى السودانيين.

اختير ثلاثون قالب شمعي لهذه الدراسة مغمورة في البرافين تم تشخيصيها مسبقا كعينات اورام البروستاتا الخبيثه, كان تمايز الورم يشمل على 6 (15%) عينة من الاورام المتباينة بشكل جيد, 9 (22.5%) عينة من الاورام المتباينه بشكل متوسط و 15(37.5%) عينة من الاورام ضعيفة التباين وعشرة قوالب كعينات اورام البروستاتا الحميدة 10 (25%).

تم قطع القوالب بسمك ثلاثة مايكرون وصبغها بطريقة الكشف النسيجي الكيميائي المناعي باستخدام تقنية البايوتين افيدين للكشف عن سايكلين د1. تم تحليل البيانات التى تم الحصول عليها باستخدام برنامج الحزم الإحصائية للعلوم الاجتماعية اصداره 16.

تراوحت اعمار المرضى بين 27 الى 90 سنة لمتوسط عمر 69 ومعظم اعمار المرضى 38 (95%) تتركز فوق 50 عاما والمريضين الباقيين (5%) اعمارهم كانت تحت او تساوي ال 50 عاما.

ظهر التعبير الايجابي ل سايكلين د1 في 30/29 عينة وسالبا في 30/1 من الاورام الخبيثة, بينما اظهرت كل عينات الاورام الحميدة تعبيرا سالبا مع وجود علاقة ذات دلالة احصائيه بين افراز سايكلين د1 ونوع الورم (القيمة الاحتمالية = 0.000).

ظهر التعبير الايجابي للسايكلين د1 في الاورام المتباينه بشكل جيد 6 (20%) و في الاورام المتباينه بشكل متوسط 9 (30%) و في الاورم ضعيفه التباين 14 (46.7%) . كما اظهر التعبير السلبي للسايكلين د1 في الورام ضعيفه التباين 1 (3.3) (القيمة الاحتمالية = 0.596).

خلصت الدراسة الى ان ظهور سايكلين د1 يزداد مع الاورام الخبيثة للبروستاتا أكثر من الاورام الحميد كما انه لا يوجد ارتباط بين ظهور سايكلين د1 و تباين الاورام.

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Chapter one

Introduction

Chapter one

Introduction

1.1 Introduction:

Carcinoma of the prostate is the most common visceral cancer in males, ranking as the most common cause of cancer-related deaths in men after carcinoma of the lung (Abbas and Mitcheals, 2007).

Worldwide prostate cancer is the second most frequently diagnosed cancer, the highest incidence rates are in United Kingdom (168.3 per 100,000) (Torre, *et al*. 2016).

In Sudan, the incidence rate of prostate cancers in Khartoum Sudan during 2009- 2010 is 13.7 per 100,000 populations (Saeed, *et al*. 2014).

The Risk factors of prostate cancer include genes mutation, age, race, heredity, environmental influences (e.g. a high-fat diet), infectious agents, smoking, vasectomy, sexual behavior, benign prostatic hyperplasia, hormonal influence and hereditary factor (Abbas and Mitcheals, 2007).

Method of prostate cancer diagnosis includes measurement of serum prostate specific antigen levels, digital rectal examination, transrectal ultrasonography, needle biopsy, immunostaining (Underwood and Cross, 2010).

Treatment of prostate cancer includes radical prostatectomy, radiation therapy, androgen deprivation therapy and chemotherapy (Rubin and Strayer, 2012).

Cyclin d1 is a key regulator of the G_1 phase progression of the cell cycle, there are increasing evidences that deregulated cyclin d1 expression is implicated in tumorigenesis and tumor progression in certain neoplasms (Drobnjak, *et al*. 2000).

Expression of cyclin d1 was assessed by Pereira, *et al* in 85 patients whose underwent radical prostatectomy for prostate carcinoma and 10 normal prostate tissue. Cyclin d1 staining was positive in 64 cases (75.4%) and negative in 21 cases (Pereira, *et al*. 2014).

Kaukoniemi, *et al* conduct study to assess the expression of cyclin d1 in clinical samples, which showed higher levels of cyclin d1 ($P = 0.0237$) and there was strong positive association between cyclin d1 and the proliferation marker Ki-67 ($P < 0.0001$) in the prostatectomy samples (Kaukoniemi, *et al*. 2015).

1.2 Objective:

1.2.1 General objective:

To detect cyclin d1 expression in prostate tumor tissue immune-histochemically and its correlation with histopathological diagnosis and tumor grade.

Chapter two
Literature review

Chapter tow

Literature review

2.1 Scientific background:

Prostatic carcinoma is the most common tumor in males. It is cancer of adenocarcinoma with a desmoplastic stroma, most often in the outer zones and posterior lobe and can be palpated on rectal examination. Tumor tends to invade nerves, seminal vesicles, and adjacent organs in the pelvis as urinary bladder and rectum and tend to metastases to the local lymph nodes and bones (Damjanov, *et al*. 2009).

2.2 Structure of the prostate:

2.2.1 Location and description:

The prostate gland lies in the pelvic cavity in front of the rectum and behind the symphysis pubis, surrounding the first part of the urethra. It consists of an outer fibrous covering, a layer of smooth muscle and glandular substance composed of columnar epithelial cells (Waugh and Grant, 2005).

2.2.2 Histology and embryology:

The prostate gland is dense organ surrounding the urethra below the bladder. It is approximately 2cm× 3cm× 4cm in size and weight about 20g. The glands are surrounded by a dense fibromuscular stroma covered by capsule. The glands are arranged in concentric layer around the urethra: the inner layer of mucosal glands, an intermediate layer of sub mucosal glands, and a peripheral layer with prostate's main glands. The prostate has three zones, cross bonding to the glandular layers: The transitional zone, the central zone and the peripheral zone (Mescher, 2010).

2.3 Disorders of the prostate:

2.3.1 Benign disorders:

2.3.1.1 Prostatitis

It's present as a variety of forms, including acute, chronic bacterial, nonbacterial and granulomatous. Acute prostatitis occurs in the setting of a urinary tract infection when infected urine refluxes into the prostate. Chronic bacterial prostatitis occurs in the context of repeated bouts of acute prostatitis or in the setting of prostatic calculi and duct obstruction. Nonbacterial prostatitis is a diagnosis of exclusion. Typically, no organism is identified and no specific therapy is available. Similarly, in granulomatous prostatitis a causative organism is frequently not identified **(**Hansel and Dintzis, 2006).

2.3.1.2 Benign nodular hyperplasia:

This common condition in middle-aged and elderly males is almost certainly due to some disturbance in the balance of male hormone production. The condition is caused by an overgrowth of the various stromal elements of the prostate, glands, smooth muscle and fibrous tissue, thereby producing glandular and stromal nodules which distort the prostatic urethra. The condition affects only the central and superior portions of the gland **(**Hansel and Dintzis, 2006).

2.3.1.3 Prostatic intraepithelial neoplasia (PIN):

It's a Premalignant changes characterized with normal architecture glands but with papillary projections into the lumen and darker staining due to hyperchromasia and enlargement of the nuclei with overlapping and stratification cells may contain pigment almost exclusively confined to peripheral zones four histologic patterns micropapillary, tufted, cribriform, flat graded as PIN I, II, or III, some use Low (I) and High (II or III) prominent nucleoli make PIN II (at least) PIN I or II can be found in 70% of "normal" patients keratin staining may be scanty, PIN III indicates up to a 70% chance of a coexisting adenocarcinoma elsewhere in the gland (Sinard, 2005).

2.3.2 Prostate adenocarcinoma:

The malignant transformation of prostate epithelial cells, as with other forms of cancer, is a result of a complex series of initiator and promoter events with genetic and environmental influences. Approximately 5 to 10% of prostate cancer cases are estimated to be related to inherited genetic factors or prostate cancer susceptibility genes. One major locus of susceptibility is found at chromosome 1q24, designated as HPC1 (hereditary prostate cancer). More than 95% of primary prostate cancers are classified as adenocarcinomas. Androgens and estrogens are two in Sudan, the incidence rate of prostate cancers in Khartoum Sudan during 2009- 2010 (the specific rate = 13.7 per 100,000) (Saeed, *et al.* 2014). hormones that appear to support carcinogenesis by influencing prostate epithelial proliferation, although elevations in androgens are not found universally in those with prostate cancer (Braun and Anderson, 2007).

2.4 Epidemiology:

There is considerable geographic variation in the age-related death rates for adenocarcinoma of the prostate throughout the world, the highest being in the United States and the Scandinavian countries, and the lowest in Mexico, Greece and Japan. Most western European countries have intermediate rates. American blacks, who exhibit a rate twice as high as white Americans, have proportionately the highest prostate carcinoma–related death rates in the world (Rubin and Strayer, 2012). In Sudan, the incidence rate of prostate cancers in Khartoum Sudan during 2009- 2010 (the specific rate = 13.7 per 100,000) (Saeed, *et al*. 2014).

2.5 Risk factors of prostate tumors:

This risk factors of prostate cancer include:

2.5.1 Hormonal influence:

Hormonal influence is further suggested by the observation that the growth of many carcinomas of the prostate can be inhibited by orchiectomy or by administration of estrogens such as diethylstilbestrol (Abbas and Mitheals, 2007).

2.5.2 Ethnic variation:

It has a wide ethnic variability, the tumor being more common on African blacks and less frequent in Hispancis and Oriental (Levison, *et al*. 2008).

2.5.3 Hereditary factor:

It has been estimated that men who have an affected first-degree relative (*e.g.,* father, brother) and an affected second-degree relative (*e.g.,* grand-father, uncle) have an eightfold increase in risk (Porth and Ravinal, 2009).

2.5.4 Age:

The vast majority of men with prostate cancer are older 50 years of age, with 75% of patients between 60 and 80 years of age (Hansel and Dintzis, 2006).

2.5.5 Genetic influences:

Much effort is focused on finding prostate cancer genes, but no definitive data are available. In studies of familial cases, several susceptibility loci on chromosome 1 have been identified. In sporadic cases, hypermethylation of glutathione *S*-transferase p1 *(GSTP1),* a genome caretaker gene on chromosome 11, and telomere shortening are relatively Common genetic alterations (Abbas and Mitcheals, 2007).

2.6 Diagnosis of prostate tumors:

There are common ways to diagnose localized or non-metastatic prostate cancer:

2.6.1 Serum prostate specific antigen:

Assay of serum levels of prostate-specific antigen (PSA) has gained widespread use in the diagnosis of early carcinoma, PSA is a proteolatic enzyme produced by both normal and neoplastic prostatic epithelium, cancer cells produce more PSA but any condition that disrupts the normal architecture of prostate including adenocarcinoma, nodular hyperplasia and prostatitis may also cause an elevation in serum level of PSA (Abbas and Mitcheals, 2007).

2.6.2 Digital rectal examination:

This is used to examine the external surface of the prostate, enlargement of the prostate due to prostatic hyperplasia usually produces a large, palpable prostate with smooth, rubbery surface. Hardened areas of the prostate gland suggest cancer and should be sampled for biopsy (Porth and Matfin, 2009).

2.6.3 Needle biopsy:

It’s an under Tran's rectal ultrasonographic guidance which may identify localized abnormalities (Underwood and Cross, 2010).

2.6.4 Biopsies:

Are frequently taken from different parts of the gland; these are referred to as sextant biopsies (Underwood and Cross, 2010).

2.7 Treatment of prostate tumors:

2.7.1 Radical prostatectomy:

The surgical removal of an enlarged prostate can accomplish by transurethral, suprapubic or perineal approach, currently transurethral prostatectomy is most commonly used technique. Prostate tissue is removed using a resectoscope and electrocautery (Porth and Matfin, 2009).

2.7.2 Radiation therapy:

Either external beam or implanted radioactive seeds (brachytherapy). In stage T3 tumors combined with androgen deprivation therapy is the treatment of choice, acknowledging that half of these patients have occult pelvic lymph node metastases (and possibly further systemic dissemination), which cannot be cured by surgical means. Patients with low-grade, low volume tumors may be managed by active surveillance only (Rubin and Strayer, 2012)

2.8 Cyclin d1 and it is relation with prostate cancer:

The d-type cyclin family, composed of three proteins, cyclin d1, d2 and D3, regulates the G1/S-phase transition of proliferating cells. Of the three D-type cyclins, it is cyclin d1 overexpression that is predominantly associated with human tumorigenesis and cellular metastases. Amplification or overexpression of cyclin d1 plays pivotal roles in the development of a subset of human cancer (Drobnjak, *et al*. 2000)

In study conducted by Pereira, *et al* who assessed cyclin d1 expression by conventional immunohistochemistry in 85 patients and 10 normal prostate tissue. Cyclin d1 staining was positive in 64 cases (75.4%) and negative in 21 cases, normal prostate tissues were negative for cyclin d1. The high-grade and low- grade Gleason score group shows ($P < 0.05$) (Pereira, *et al*. 2014). Kaukoniemi, *et al*. Study the expression of cyclin d1 in clinical samples, they performed an immunohistochemical analysis which showed that the samples expressed higher levels of cyclin d1 (*P* =0.02). In prostatectomy samples, the cyclin d1 staining intensity was not associated with Gleason score, T stage, or diagnostic PSA levels. However, there was strong positive association between cyclin d1 and the proliferation marker Ki-67 (P < 0.0001) in the prostatectomy samples (Kaukoniemi, *et al*. 2015).

Chapter three

Materials and methods

Chapter three
Materials and methods

3.1 Materials:

Archived tissue blocks of prostate tumors were selected for this study.

3.2 Methods:

3.2.1 Study design:

This is a hospital based analytical retrospective case control study aimed to detect cyclin d1expression in prostate tumors.

3.2.2 Study samples:

Tissue blocks obtained from thirty samples previously diagnosed as malignant prostate tissue and ten samples which previously diagnosed as benign tumor were collected. Patient's data (age, histopathological diagnosis) were obtained from patient’s files.

3.2.3 Study area:

This study was held in Omdurman hospital laboratory during the period from July 2017 to November 2017.

3.2.4 Sample processing:

Section of 3µm thickness were cut by rotary microtome, mounted in positively charged glass slides and put at 60℃ oven for 30 minutes.

3.2.5 Immunohistochemical staining:

Immunohistochemical staining was carried out using indirect streptoavidin –biotin immune peroxidase technique. Tissue section (3µm) were deparaffinized in xylene and rehydrate in graded alcohol (100% 90%, 70%, 50%) and water two minutes for each, then slides were incubated for 10 minutes in 0.3% hydrogen peroxide to block endogenous peroxidase activity. Antigen retrieval was performed by using PT link water path with citrate buffer (Ph 6.8). The slides were then treated with anti-cyclin d1 primary antibody for 30 minutes, then sections were incubated in biotinylated secondary anti body for 15 minutes then washed in phosphate buffer saline (Ph 7.4), incubated in streptoavidin- HPR (horse radish peroxidase) for 15 minutes, washed in phosphate buffer saline (ph 7.4), incubated in diamainobenzidine tetrahydrochlorid (DAB) substrate solution, washed in running tap water. Then counter stained in Mayer's hematoxylin stain for 1minute. Dehydrated in grading alcohol (50%, 70%, 90%, and 100%), cleated in xylene, and mounted in DPX mounted media (Bancroft, *et al*. 2013).

3.2.6 Data analysis:

Data analysis was done using SPSS 16 computer program. Frequency mean and chi- squared test value were calculated.

3.2.7 Result interpretation:

All quality control measure was adopted, positive and negative control slides were used during immunohistochemical staining. Detection of more than 5 cells with cytoplasm per one field considered as positive result.

3.2.8 Ethical consideration:

Samples were collected after tacking ethical acceptance from hospital administration.

Chapter four

Results

Chapter Four

Results

In this study thirty blocks of previously diagnosed as prostatic adenocarcinoma and ten blocks of prostatic hyperplasia were used.

The age of study population ranges between 27 and 90 years with mean age 69 years, most of patients 38 (95%) were more than 50 years and the remaining 2(5%) were less or equal 50 years (table 4.1).

The differentiation of malignant samples was as follow: 6 (15%) samples were well differentiated prostate cancer, 9 (22.5%) samples were moderately differentiated prostate cancer and 15 (37.5%) samples were poorly differentiated prostate cancer (table 4.2).

Positive expression of Cyclin d1 was not found among benign prostate tumor 0/10 (0.0%) samples and in malignant samples the expression was 29/30 (72.5%) samples. While the negative expression of Cyclin d1 among benign prostate tumor samples 10/10 (25%) and in malignant tumor samples 1(2.5%) (P. value 0.000) (table 4.3).

The positive expression of Cyclin d1 on tumor differentiation was as fallow: well differentiated tumor 6/6 (20%), moderately differentiated tumor 9/9 (30%) prostate tumor and on poorly differentiated tumor was 14/15 (46.7) and negative expression on poorly differentiated tumor was 1/15 (3.3) (P. value 0.596) (table 4.4).

Table (4.1): Distribution of age group among study samples population:

Age group (years)	Frequency	Percent
Less/equal 50 years	2	5%
More than 50 years	38	95%
Total	40	100%

Table (4.2): Distribution of histopathological diagnosis among study samples:

Histopathological diagnosis		Frequency	Percent
Malignant	Well differentiated tumor	6	15%
	Moderately differentiated tumor	9	22.5%
	Poorly differentiated tumor	15	37.5
Benign	Benign prostatic hyperplasia	10	25%
	Total	40	100%

Table (4.3): Relation between Cyclin d1 expression and histopathological diagnosis of prostate tumor:

Histopathological diagnosis	Expression of Cyclin d1		Total	P.value
	Positive (%)	Negative (%)		
Benign	0(0.0%)	10(25%)	10(25%)	0.000
Malignant	29(72.5%)	1(2.5%)	30(75%)	
Total	29(72.5%)	11(27.5%)	40(100%)	

Table (4.4): Relation between Cyclin d1 expression and prostate cancer differentiation:

Tumor differentiation					P. value
Expression of Cyclin d1	Well differentiated	Moderately Differentiated	Poorly differentiated	Total	
Positive	6(20%)	9(30%)	14(46.7)	29(96.7%)	0.596
Negative	0(0%)	0(0%)	1(3.3)	1(3.3)	
Total	6(20%)	9(30%)	15(50%)	30(100%)	

Photo (4.1): Benign prostatic hyperplasia tumor show negative nuclear expression for Cyclin d1 (40X).

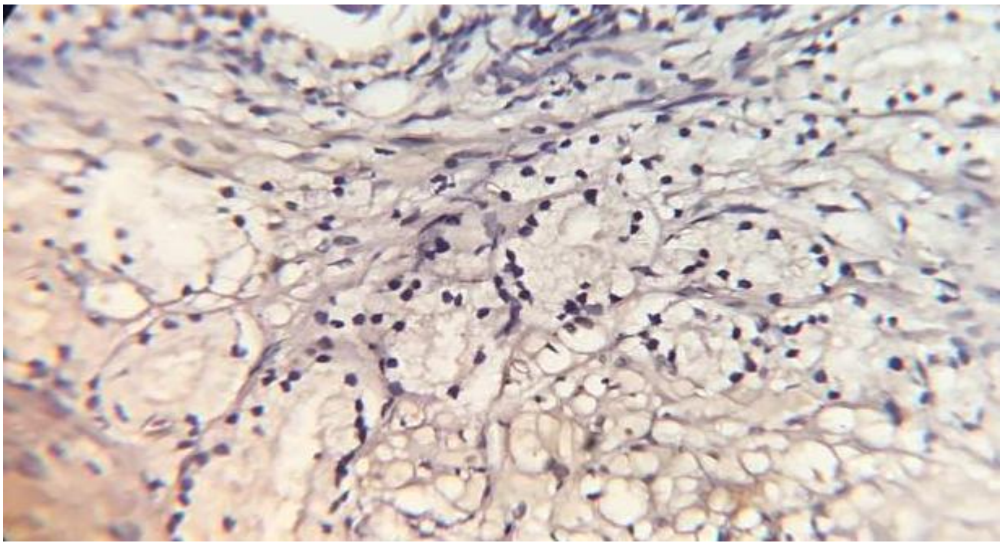
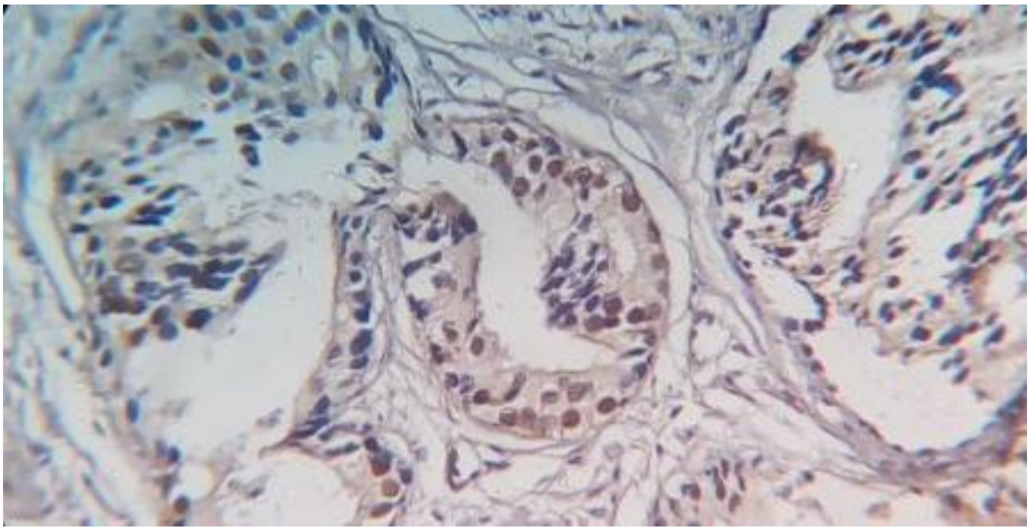


Photo (4.2): Malignant prostatic tumor show positive nuclear expression for Cyclin d1 (40X).



Chapter Five

Discussion and recommendations

Chapter Five

Discussion

5.1 Discussion:

The current study aimed to immunohistochemically detection of cyclin d1 in prostate tumor among Sudanese patients.

Regarding the age of the patients the study revealed that most of patients were more than 50 years. This finding meanings that older individuals are more susceptible to prostate tumor than younger, this results compatible with Pereira *et al.* (2014), who reported that the prostate cancer more common in elder adult with mean age 67 years old.

The positive expression of Cyclin d1 among prostatic carcinoma was showed significant correlation (P. value 0.000) and showed high frequency of poorly differentiated prostatic carcinoma, that means our results of the Cyclin d1 expression among examined prostate carcinoma cases, showed marked characteristic of full expression within most of the cases This finding has been found in agreement with Gupta *et al*. (2014) who reported that a significant expression of Cyclin d1 among prostatic carcinoma was 100%. If it is Also compatible with Ozbek *et al*. (2000) who reported that the expression of cyclin d1 was positive in all prostate cancers cases.

In our study we found that all benign prostatic hyperplasia assessed showed no immunostaining for cyclin d1, this was in agreement with study conducted by Pereira *et al.* (2014) and Comstock *et al.* (2007), they found low or absent cyclin d1 expression in benign prostatic hyperplasia.

On other hand not compatible with Ueda *et al*. (2001) who found that 53.8% of cases of benign prostatic hyperplasia showed cyclin d1 expression.

5.2 Conclusion:

On basis of this study we conclude the follow:

- The age of the prostate cancer among study group is commonly more than 50 years.
- Most differentiation of prostate cancer – in the study samples – is poorly differentiated cancer.
- Cyclin d1 positive expression is associated with malignant prostate tumor.
- Cyclin d1 positive expression has no association with tumor grade.

5.3 Recommendations:

On basis of this study we recommend the follow:

- Further study using other approaches to elucidate the efficacy of Cyclin d1 as tumor marker of therapeutic target in prostate cancer.

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Appendices

Instrument and Material:

Instrument:

Disposable gloves.

Rotary microtome.

Microtome Knives.

Coplin jars.

Oven.

Staining racks.

Coated slides.

Water path.

Cover glass.

Dako pen.

Humidity chamber.

Materials:

Mayer`s haemtoxylin .

Xylene.

Ethyl alcohol (absolute,90%,70%,50%).

Distilled water.

Peroxidase blocker .

Primary antibody (Cyclin d1).

Secondary antibodies (biotinylated secondary antibody).

3.3 di amino benzidine tetra hydrochloride in substrate buffer.

DPX mounting media.

Phosphate (PH 7.4) component:

Solution A (0.2 M sodium di hydrogen orthophosphate, 3.12g di sodium hydrogen orthophosphate, 100 ml DW).

Solution B (0.2 M sodium di hydrogen orthophosphate, 2.83g di sodium hydrogen orthophosphate, 100ml DW) (9.5ml from solution A +40.5ml solution B).

Citrate buffer (PH6.8) component:

Solution A (0.2M sodium di hydrogen orthophosphate, 2.83g di sodium hydrogen orthophosphate, 100ml DW).

Solution B (2.1g citric acid, 100ml DW)(27.7ml from solution A+22.8ml from solution B).

Mayer`s haematoxlin component:

Haematoxlin powder	1 gm
Potassium alum or ammonium alum	50gm
Sodium iodate	0.2gm
Citric acid	1 gm
Chloral hydrate	50gm
Distilled water	1000ml

Ammoniated water:

Concentrated ammonia	0.05ml
Tap water	99.95 ml