A Biochemical Study for Evaluation and Analysis of Serum Protein of Patients with Different Kidney Tumors

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Abstract:
The amount of protein in the serum depends on the balance between the rate of its synthesis, and that of its catabolism or loss. Abnormal metabolism may result from nutritional deficiency, enzyme deficiency, abnormal secretion of hormones, or the actions of drugs and toxins. Renal cancer is the third most common malignancy of the genitourinary system, and accounts for 3% of adult malignancies globally. Total serum proteins were measured in malignant kidney tumor, benign kidney tumors, and non tumoral kidney diseases patient groups, as well as in healthy individuals. A significant decrease ($p< 0.001$) of total serum protein levels in patients with malignant kidney tumors when compared with those of benign tumors, non tumoral diseases, and healthy individuals. The lowest serum protein levels were found in patients with stage IV, regardless their genders. Analysis of total serum proteins using PAGE revealed clear differences in the number and shape of the bands in patients with different kidney diseases compared with healthy controls.

Key words: Serum proteins, Kidney, Tumors

Introduction

Renal cancer is the third most common malignancy of the genitourinary system [1], and accounts for 3% of adult malignancies globally [2]. The incidence of renal cell carcinoma (RCC) has been increasing 2to4% per year since the 1970s, perhaps, it related in part to the improvement and increased use of modern imaging techniques [3]. Limited early warning signs result in late recognition with metastases present in approximately one third of patients at the time of diagnosis [4], with 210,000 new cases per year and more than 100,000 deaths occurring worldwide annually. The male to female ratio is 1.5:1, and the disease usually occurs in the sixth and seventh decades of life[5]. RCCs are highly vascularized tumors, which may explain the 30-40% prevalence of metastatic disease at initial diagnosis [6,7], when systemic therapies are then necessary. In this group of patients, one-year survival rate are ~25%, illustrating the limited role of both chemotherapy and radiotherapy in the management of advanced stages of RCC[8]. Advanced RCC responds poorly to systemic therapy and has a 5-year survival rate of less than 10% [9,10].

The amount of protein in the serum depends on the balance between the rate of its synthesis, and that of its catabolism or loss. Abnormal metabolism may result from nutritional deficiency, enzyme deficiency, abnormal secretion of hormones, or the actions of drugs and toxins[11]. Cancer patients often present to their physicians in a poor nutritional state. Their nutritional deficits have a

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significant impact on mortality, morbidity and quality of life [12]:

Materials & Methods

1) Patients and Control Individuals

During the period from the beginning of February 2007 to the end of July 2008, 96 patients of different tumoral and non tumoral kidney diseases in addition to 46 healthy individuals were enrolled in the present study. The kidney patients group was subdivided into three subgroups, i.e., malignant kidney tumors, benign kidney tumors, and non tumoral kidney diseases, their informations are illustrated in table 1.

Table 1: Host Informations of the Kidney Patients Groups

Kidney Patient Groups (n.) Age Range (year)	Gender	
	Male (n.) Age Range (year)	Female (n.) Age Range (year)
Malignant (55) (32 – 80)	36(32-80)	19(37-65)
Benign (23) (10 – 62)	14(10-66)	9(25-62)
Non Tumoral Affections (18) (12 – 68)	11(27-62)	7(12-68)

2) Serum Samples:

Ten milliliters of venous blood samples were collected from patients and the control groups. Samples were allowed to clot at room temperature, centrifuged at 3000 xg for 5 minutes, then sera were collected and stored at -15°C.

3) Determination of Total Serum Proteins Levels

A total serum protein was estimated using Biuret method [13]. The bovine serum albumin was used as a standard protein. The procedure was carried out as in the following:

Test	Blank
1) 50 µL serum or standard	**1)** 50 µL distilled wate
2) 2.5 mL Biuret reagent	**2)** 2.5mL Biuret reagent
3) The mixtures were incubated at room temperature for 30 minutes	
4) The absorbance was measured at 540 nm	

4) Conventional Polyacrylamide Gel-Electrophoresis (PAGE)

The application note 2117 issued by LKB company was used for the application of, conventional polyacrylamide gel electrophoresis, to separate proteins and glycoprotein as in the following:

a. Polyacrylamide gel (separating gel with concentration of 7.5%) was prepared by mixing the following solutions (66 mL total volume), respectively:

Reagents	Amount
Distilled water	7.5 mL
Tris-HCl -glycine buffer stock solution (0.15 M, pH 8.9)	33 mL
Polyacrylamide solution 7.5%	22.2 mL
Ammonium persulphate 1.5% (w/v)	3.2 mL
N, N, N`, N`-tetramethyl ethylene diamine (TEMED) (2.6%)	0.1 mL

The mixture was de-gassed in a 250 mL vacuum flask for 15 minutes then 1.5% ammonium persulphate was added.

b. The mixture was gently mixed and immediately poured into the mould up to the mark delineating the separation gel, then butanol was added for 1cm above the gel to prevent oxygen dissolvation into the gel. When the polymerization reaction was completed (within 40 minutes). The mould was put in the refrigerator for further 15 minutes.

c. Buffer tanks of the multiphor were filled with the electrode buffer, 1L for each tank. The gel glass plate was placed on the cooling plate and connected with the buffer by means of one layer of electrode wicks on both sides. They were soaked in the buffer, and then placed on the edge of the gel, covering 10-12 mm.

d. Pre electrophoresis was carried out at 50 mA and 15 v/cm for 30 minutes according to

application note 306 of LKB Company.

e. A volume of 10 µL of samples were applied into application zones of the gel, and concentrated for 5-10 minutes. With a current of 20 mA, electrophoresis was continued using 40 mA, until the blue color of the bromophenol blue dye reached the second side of the gel.

The gel was divided into two parts; one for protein staining, and the second for glycoprotein staining

Reagents:

Stock Tris-glycine Buffer (0.15 M, pH 8.9): Prepared by dissolving 22.89 g of glycine in a 1L of distilled water. The pH of the solution was adjusted to pH 8.9 by the addition of Tris (hydroxy-amino methane). The volume was made up to 2 liters with distilled water. Working Tris-glycine Buffer (Electrode buffer): Prepared by diluting one part of the stock Tris-glycine Buffer (0.15 M, pH 8.9) solution with an equal volume of distilled water. Polyacrylamide Solution 7.5%: a weight of 22.2 g of acrylamide and 0.6 g of N, N`-methylene bisacrylamide, were dissolved in 60 mL distilled water and then the volume was made up to 100 mL with distilled water. Ammonium Persulphate 1.5% (w/v): was prepared by dissolving 150 mg of ammonium persulphate in 10 mL of distilled. This solution was prepared on day of the use. Bromophenol blue 0.25% (w/v): Prepared by dissolving of 0.25 g of bromophenol blue in 100 mL of Tris - glycine Buffer (an electrode buffer). N, N, N`, N`-tetramethyl ethylene diamine (TEMED) (2.6%)

5) Gel Staining Methods

a) Protein Staining:

The polyacrylamide gel was stained for protein, using Silver Stain as follows: The gel was placed into the fixing solution (solution 1) for 2 hours or overnight. After fixation, the gel was washed 3 times with washing solution (solution 2) for 20 minutes. For sensitization, the gel was soaked in sensitizing solution (solution 3) for 2 minutes. The gel was washed 3 times with distilled water for 5 minutes for each. Then, the gel was soaked in the staining solution (solution 4) for 20 minutes. After staining, the gel was washed 2 times with distilled water for 1 minute. The color was developed by the developing solution (solution 5) until the band being clear. For stopping color development, the gel was soaked in stopping solution (solution 6) for 5 minutes. The gel was left at 4 °C in 1% glacial acetic acid.

Reagents:

Fixing Solution (Solution 1): Prepared by mixing of 50 mL ethanol (95%) with 12% glacial acetic acid and 20 µL of 37% formaldehyde, the volume was completed to 100 mL with distilled water. **Washing Solution (Solution 2):** Prepared by completing of 50 mL of ethanol (95%) to 100 mL with distilled water. **Sensitizing Solution (Solution 3):** Prepared by dissolving of 0.02 g of sodium thiosulphate in 100 mL of distilled water. **Staining Solution (Solution 4):** Prepared by mixing of 200 mL of 0.2 % silver nitrate and 152 µL of 35% formaldehyde. **Developing Solution (Solution 5):** Prepared by mixing of 400 mL of 6% sodium carbonate with 8 mL of 0.02% sodium thiosulphate, and 200 µL of 35% formaldehyde. **Stop Solution (Solution 6):** Prepared by mixing of 200 mL of 50% methanol and 12% glacial acetic acid.

b) Glycoprotein Staining:
The slab gel was immersed in the fixing solution (solution 1) for one hour, then washed with distilled water for 1 minute. The gel was immersed in staining solution (solution 2) for 30 minutes, then put in distilled water for 24 hours (water was changed several times). The slab gel was put in the oxidation solution (solution 3) in a dark place for 50 minutes, the gel was washed with washing solution (solution 4) until the bands of the glycoproteins appear. The slab gel was immersed in the preserving solution (solution 5). Using this procedure, the bands were stable for several days.

Reagents:
Fixing Solution (Solution 1): Prepared by dissolving 12.5 g of trichloroacetic acid in 100 mL of distilled water. **Staining Solution (Solution 2):** Prepared by dissolving of 1 g of Basic Fuchsine in 200 mL of distilled water with heating. After cooling, 20 mL of HCl (1N) and 2 g of sodium metabisulphate were added with stirring. The solution was left for 6 hours at 4 ºC, then 2 g of activated charcoal was added. The solution was filtered using Whitman paper No.1. **Oxidation Solution (1%) Periodic Acid–3% Acetic Acid) (Solution 3):** Prepared by mixing 1mL of periodic acid and 3 mL of acetic acid in a final volume of distilled water. **Washing Solution (0.5 % in Sodium bisulphide and 0.5 M HCl) (Solution 4):** this solution was prepared by dissolving 500 mg of sodium bisulphide in 100 mL of 0.5 M of HCl. **Preserving Solution (Solution 5):** (10% Glycerol)

Results and Discussion:

➤ **Levels of Total Serum Proteins in Kidney Patients and Healthy individuals Groups:**

Total serum proteins were measured in malignant and pathological control group patients, as well as in healthy individuals. Table 2, shows a significant decrease ($p < 0.001$) of total serum protein levels in patients with malignant kidney tumors when compared with those of benign tumors, non tumoral diseases, and healthy individuals. Non significant variations were observed during the comparison of the group of benign kidney tumors with those of non tumoral diseases as well as for the group of benign kidney tumors with the group of healthy individuals. Moreover, non significant variations was found when non tumoral kidney diseases were compared with healthy individuals group.

Table 2: Total Serum Protein Levels (g / dL) in Tumoral (Malignant and Benign) and Non Tumoral Kidney Patients and Healthy Individuals

Groups	Age (year) Mean ± S.D. Range	TSP Mean ± S.D.	Range	p
K_1(55)	54.93 ± 12.50 32 – 80	4.70 ± 0.82	3.14 – 6.26	0.000**for K_1 vs K_2
K_2(23)	45.04 ± 15.33 10 – 66	6.17 ± 0.88	4.40 – 7.57	0.000**for K_1 vs K_3
K_3(18)	42.39 ± 16.60 12 – 68	5.92 ± 0.82	4.58 – 7.28	0.349 for K_2 vs K_3
H_{K1}(32)	47.38 ± 10.92 32 – 80	5.75 ± 0.76	4.35 – 7.40	0.000**for K_1 vs H_{K1}
H_{K2}(43)	39.77 ± 13.77 10 – 66	6.01 ± 0.73	4.35 – 7.40	0.451 for K_2 vs H_{K2}

K_1: Malignant Kidney Tumor Patient group, K_2: Benign Kidney Tumor Patient group, K_3: Non Tumoral Kidney Patient group, H_K: healthy individuals for comparison with Malignant Tumor Patients, H_{K2}: healthy individuals for comparison with Benign Tumor and Non Tumoral Kidney Patients. TSP values are represented as Mean ± S.D. The mean difference is significant at the 0.001 level. **Refer to highly significant differences between the variables

➢Relevance of Total serum Protein Levels with Stages of Malignancy:

To verify the relevance of the aggressiveness of malignancies of kidney with serum protein level changes, the analysis of variance (ANOVA) test was used for the evaluation of the data of the 4 stages (I, П, Ш, and IV) together. Table 3 indicates to non significant difference was noticed when malignant kidney tumors with stage I was compared with those of stage II. On the other hand, significant variations ($p < 0.001$) were found when the malignant kidney tumors patients with stage I was compared with other kidney tumors stages. Non significant differences were noticed when stage II of the malignant kidney tumors group were compared with other stages patients groups (III and IV). When the two advanced stages were compared together, non significant difference was demonstrated

Table 3: Comparison of Total Serum Proteins (TSP) (g /dL) in Different Malignant Kidney Tumor Stages

Subjects	Age (year) Mean± S.D. Range	TSP Mean± S.D.	Range	p
Stage I(14)	49.07 ± 11.94 32 – 74	5.44 ± 0.61	4.28 – 6.26	0.089 for (1)
Stage П(12)	55.67 ± 13.85 34 – 79	4.93 ± 0.58	3.55 – 6.08	0.000** for (2, 3)
Stage III(11)	53.73 ± 9.71 43 – 75	4.24 ± 0.62	3.18 – 5.42	0.030 for (4)
Stage IV(18)	59.72 ± 12.40 41 – 80	4.26 ± 0.77	3.14 – 5.76	0.017 for (5)
				0.956 for (6)

TSP values are represented as Mean ± S.D. The mean difference is significant at the 0.001 level.*Refer to significant difference between the variables. **Refer to highly significant between the variables

1) Stage I vs Stage II
2) Stage I vs Stage III
3) Stage I vs Stage IV
4) Stage II vs Stage III
5) Stage II vs Stage IV
6) Stage III vs Stage IV

➢Gender Differences of Total Serum Proteins Levels in Kidney Diseases Patients:

Patients of malignant tumors of kidney group were classified into two groups (males and females). Males constituted 65 % of patients and females were 35 % of those patients. Male patient percentages were also higher in other groups of kidney tumor patients. No significant variations were obtained among the comparison with male and female patients (table 4).

Table 4: Comparison of Total Serum Proteins (g / dL) in Male and Female of Malignant and Benign Tumors, Non Tumoral Kidney Affections Patients, and Healthy Individuals

Type	Gender	Age (year) Mean ± S.D. Range	TSP Mean ± S.D.	Range	p
K_1 (55)	M(36)	57.31 ± 13.69 32 – 80	4.60 ±0.86	3.14 – 6.26	0.205
	F(19)	50.79 ± 9.19 37 – 65	4.90 ± 0.74	3.85 – 6.15	
K_2 (23)	M(14)	43.93 ± 16.73 10 – 66	6.23 ± 0.87	4.40 – 7.39	0.644
	F(9)	47.44 ± 12.28 25 – 62	6.07 ± 0.95	4.97 – 7.57	
K_3 (18)	(M11)	47.36 ± 11.33 27 – 62	5.82 ± 0.70	4.98 – 7.05	0.530
	(F7)	34.57 ± 21.22 12 – 68	6.07 ± 1.03	4.58 7.28	
H (46)	M(21)	44.24 ± 9.57 10 – 81	5.85 ± 0.67	4.90 – 7.40	0.396
	F(25)	44.88 ± 17.10 11– 87	5.77 ± 0.86	4.35 – 7.07	

K_1: Malignant Kidney Tumor Patient group, K_2: Benign Kidney Tumor Patient group, K_3: Non Tumoral Kidney Patient group, and H: healthy individuals. M: Male, F: Female. TSP values are represented as Mean ± S.D.

In the malignant tumors patients groups, a decreased in the total sera and tissues proteins levels with the tumor progression stages were like each other in the different cancers study's groups (data non shown).

➤**Analysis of Total Serum Proteins of Patients with Tumoral and Non Tumoral Kidney Diseases and Healthy Individuals:**

Total serum proteins were analyzed by polyacrylamide gel and stained with **Silver** and **Periodic acid stains**. Albumin (band number 8), α_1 (band number 7), α_2(band number 6), β (band number 5), and γ-globulins (bands number 4 ,3, and 2), which formed the five main groups of proteins in the standard normal serum electrophoregram profile as mentioned by Yang et al [14] were present in the tested crud sera samples.

Sera of the healthy individuals group contained about 10 protein bands with different mobilities and molecular masses. The electrophoresis pattern of non tumoral kidney affection group reflect the same bands profile as that of the healthy individuals group, with a clear difference in the width of the high molecular weight proteins bands (bands number 2, 3, and 4); especially, that refers to the increase in these proteins concentrations and (or may be refers to) alteration in their structures. While the lower molecular weight proteins bands (band number 9 and 10) in pattern of non tumoral kidney group identified their corresponding in healthy sera group in both number and shape.

In the pattern of benign kidney tumors sera electrophoresis, the noticeable reduction in the intensity and width of albumin band (band number 8) when it compared with their similar in non tumoral kidney diseases and healthy groups, moreover; presence of an additional band (band number 11; which appeared only in the benign and malignant kidney groups, however; they are different in their size and shape), they were characterized benign tumors electrophoresis pattern. 2, 3, and 4 bands were interlaced and so close together, where they appeared as a single band. Increase and decrease in the bands number and intensity of the benign kidney tumor pattern may explain the cause about the normal levels for total proteins in sera of benign tumor patients.

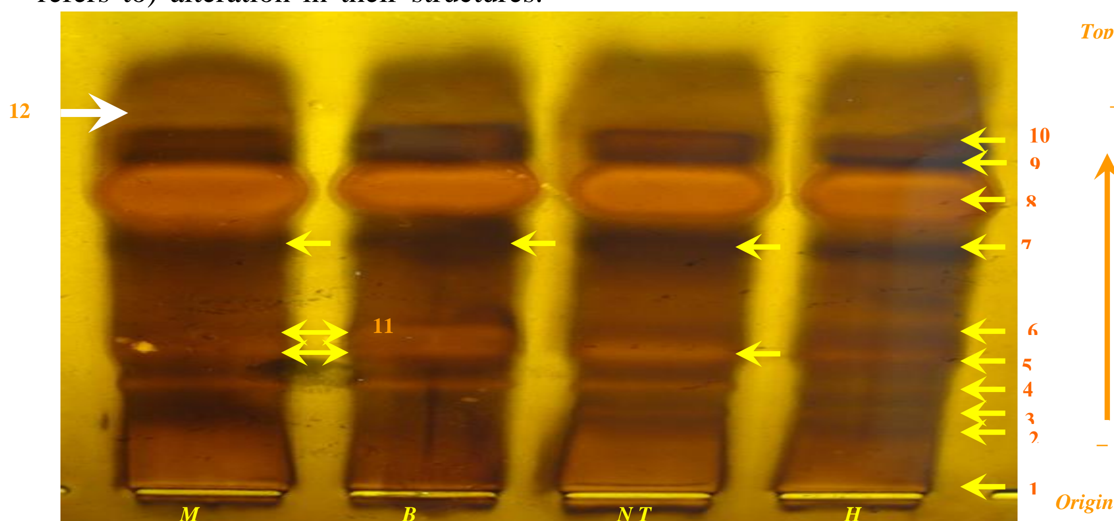

Fig.1: Conventional Polyacrylamide Gel Electrophoresis (PAGE) 7.5% for Proteins. Tris - glycin buffer (0.075 M, pH 8.9) used as the electrodes buffer solution. Prelctrophoresis conditions were 50 mA as a constant current for 30 minutes and voltage of 15 v/cm. Electrophoresis was carried out for 10minutes at 20 mA, then the process was performed for 3.5 hours at 4ºC by using 40 mA as a constant current and voltage of 15 v/cm. The gel was stained for protein with silver. The crude samples that applied were:

M: Sera of Malignant Kidney Tumors Patients ***B: Sera of Benign Kidney Tumors Patients***

NT: Sera of Non Tumoral Kidney Affections Patients ***H: Sera of Healthy Individuals***

The electrophogram pattern of malignant kidney tumors sera is characterized by presence of significant reduction in the proteins bands intensity in comparison with other studied groups, except albumin band (band number 8) in which obvious increase was noticed. In the γ-globulins region (bands number 2, 3, and 4) an overlapping among the bands was noticeable, where they appeared, rather; as a blot. On the other hand, the malignant group had only a single band (band number 12) at the positive end of the gel, which may be referring to the presence of different protein(s) in the malignancy case only. Decrease in the malignant sera proteins pattern may justify the decrease in the total serum proteins levels of the malignancy patients.

Periodic acid stain of crude pooled sera of patients and the control group revealed qualitatively incompatible electrophoretic pattern in particular for patients with malignant tumors (figure 2). The additional bands that appear in glycoprotein profile, particularly in the globulins region due to the changes in cell surface glycoproteins during malignancy, and (or) as a result of synthesis of the acute phase proteins during inflammation processes [15].

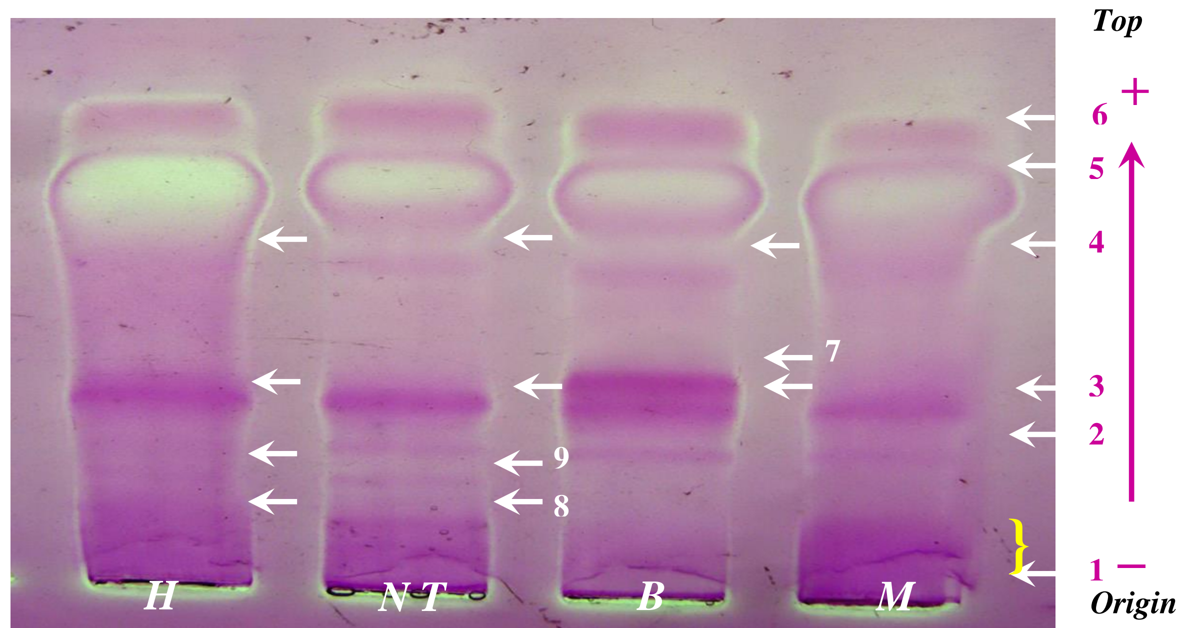

Fig.2: Electrogram of Glycoproteins profile using Conventional Polyacrylamide Gel Electrophoresis (PAGE) 7.5%. Tris - glycin buffer (0.075 M, pH 8.9) was used as the electrode buffer. Prelctrophoresis conditions were 50 mA as a constant current for 30 minutes, with voltage of 15 v/cm, and at 4 ºC. Electrophoresis was carried out for10 minutes at 20 mA. The process was continued for 3.5 hours at 4 ºC by using 40 mA as a constant current and voltage of 15 v/cm. The gel was stained for protein with periodic acid-Schiff. The crude samples that applied were:

M: Malignant Kidney Tumors Sera ***B: Benign Kidney Tumors Sera***

NT: Non Tumoral Kidney Affections Sera ***H: Healthy Individuals Sera***

There is a growing interest in testing the hypothesis that the blood, serum, and tissue proteome contain protein biomarkers that are useful for classifying the physiological or pathological status of an individual. Such markers are expected to be useful for the prediction, detection, classification and diagnosis of disease as well as to follow the efficacy, toxicology, and side effects of drug treatment. In addition to that, a distractive serum proteins profile involving relatively abundant proteins

may be observed in cancerous cases relative to healthy subjects or patients with chronic diseases [16].
In the present study, the decrease of the total serum and tissue proteins of patients with cancerous diseases can be explained by several hypotheses: **(A)** The first is that the deficiency of total serum proteins during carcinogenesis may be involved in the feedback control of enzyme system required for cell division [15]. It has been found that one of the most important alterations in the cancerous cell is an increased level of enzymes involved in nucleic acid synthesis and production of lytic enzymes, e.g., proteases, collagenases, glycosidases. An increase in the advancing of the stage of the malignant tumor cells and the increase in the tumor size will be associated with the increase in the catabolic pathways of the normal cells to supply malignant cells with the primary materials for their propagation, and on the other side to produce the oncoproteins. If this assumption be right, several proteins levels in sera and tissues will be disorganized (may decrease) that lead to decrease in total proteins levels. **(B)**The second hypothesis is the notion that the proteolytic system and its substrates may reside in the same compartment led to the prediction that a tightly controlled, energy-dependent, and highly specific system must be involved in their degradation [17, 18]. The discovery of the ubiquitin-proteasome pathway has resolved these enigmas. Naturally, in the protein degradation process many essential steps are involved as shown in figure 3. It appears that two distinct and unrelated groups of proteins determine the specificity of the ubiquitin system: **(1)** E3s and **(2)** ancillary proteins. Within the ubiquitin system, substrates must be specifically recognized by an appropriate E3 as a prerequisite to their ubiquitination.

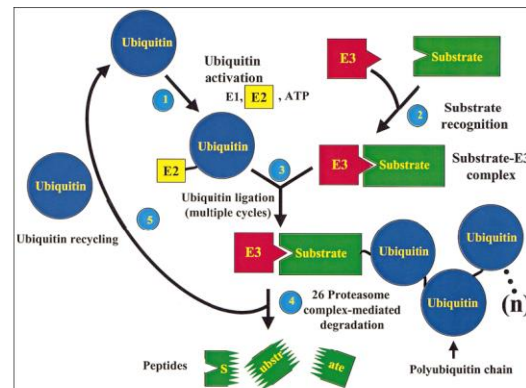

Fig.3: Degradation of a protein via the ubiquitin pathway [19].

In most cases however, substrates are not recognized in a constitutive manner. They must undergo a posttranslational modification, phosphorylation for example, or associate with an ancillary protein, a molecular chaperone for example, to be recognized by the ligase. Similarly, in other cases, the E3 is switched on by undergoing post-translational modification or association with a specific activator to yield an active form that recognizes the substrate [19]. Some pathologies result from uncontrolled, accelerated removal of a substrate (loss of function) [20], cancer is one of these diseases, since several malignancies have been attributed to aberrations in the ubiquitin pathway [21]. Thus, the studied patients of malignant diseases may suffer from ubiquitin system disorders that may cause increase in the enzymatic activity of the ubiquitin pathway. **(c)** The third hypothesis one is that malignancies are frequently associated with active catabolic pathway and negative nitrogen balance [15].
The current study results were in agreement with several previous studies dealt with the evaluation of serum and/or tissue proteins in bladder cancer [22], gynecologic malignancies [23], breast cancer [24-26],

gastrointestinal tract cancers [27], and head and neck cancer [12]. In these studies, a trend of a decrease in serum and / or tissue proteins was observed. On the other hand, the present results were in contrast with Wei-Min [16] study. They found significant increases in several protein profiles levels sera of lung cancer patients, using protein microarray and immunoassay. In bone tumors study by Al-Gazally [28], in the oral cavity tumors study by Daoud [29], and the study of the different cancers types (colon, stomach and breast) by Al-Barqawii [30] a significant increase in total serum and tissue protein levels was found in cancer patients.

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دراسة كيموحيوية لتقييم وتحليل البروتينات المصلية لدى مرضى الأورام الكلوية المختلفة

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الخلاصة:

كمية البروتين في المصل تعتمد التوازن بين نسبة التخليق ومقدار ما يؤيض منه هدمياً. الايض غير الطبيعي للبروتين قد يكون ناجماً عن نقص التغذية أو نقصاً إنزيميا أو عن افرازغير طبيعي للهرمونات أو نتيجة لفعل دوائي أو سمي. سرطان الكلية هو ثالث سرطانات الجهاز البولي شيوعاً ويشكل 3% من مجمل حالات السرطان. تم قياس مستويات البروتين الكلي في أمصال مرضى الأورام الكلوية الخبيثة منها والحميدة ومرضى الاعتلالات الكلوية غير الورمية وكذلك لدى الأفراد الأصحاء، وقد لوحظ انخفاض معنوياً ($p < 0.001$) في مستوى البروتينات المصلية عند مرضى الأورام الكلوية الخبيثة مقارنة مع أفراد باقي المجاميع، وقد سجلت أوطأ مستوياته عند مرضى المرحلة الرابعة من الورم بغض النظر عن جنس المريض.تحليل البروتينات الكلية باستخدام تقنية الترحيل الكهربائي على هلام الاكريل امايد لأمصال مجاميع الدراسة اظهر اختلافاً واضحاً في أعداد وأشكال الحزم عند مرضى الاعتلالات الكلوية مقارنة مع الأفراد الأصحاء.