ISSN: 0067-2904
GIF: 0.851

Assessment the Immunohistochemical Expression of Cathepsin D in Iraqi Patients with Colorectal Carcinoma

Ban Jasim Mohamad[1*], Khawla Hori Zghair[1], Faeza Aftan Zghair[2], Reyadh Salim Mohammed[3]

[1]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq
[2]Ibn-Sina College of Medicine, Al-Iraqia University, Baghdad, Iraq
[3]Department of Biology, College of Science, University of Tikrit, Tikrit, Iraq

Abstract:

In the current study hundred ten of Iraqi patients with colorectal tumors were studied to evaluate the expression of Cathepsin D using Tissue microarray-Immunohistochemisty (TMA-IHC) technique. Of them, Ninety cases had colorectal carcinoma, and twenty had benign tumors. A group of twenty cases of non-specific colitis and other twenty colonic biopsies without significant pathology were also studied. Results revealed high expression of Cathepsin D in tumors (96.7% of malignant cases, 80% of adenomas) versus 30% of colitis. As well high significant differences (P< 0.001) in the expression of CathepsinD in different tumor types (adenocarcinoma and mucinous carcinoma), tumor stages and grades.

Keywords: BRAF, $BRAF^{V600E}$, colorectal cancer

تقييم التعبير الكيميائي النسيجي المناعي لل Cathepsin D في المرضى العراقيين المصابين بسرطان القولون والمستقيم

بان جاسم محمد[*1]، خولة حوري زغير[1]، فائزة عفتان زغير[2]، رياض سالم محمد[3]

[1] قسم علوم الحياة، كليةالعلوم، جامعة بغداد، بغداد، العراق
[2]كلية طب ابن سينا، الجامعة العراقية ، بغداد، العراق
[3]قسم علوم الحياة ، كلية العلوم، جامعة تكريت، تكريت، العراق

الخلاصة

هدفت هذه الدراسة الى تقييم التعبير الكيمياءي المناعي النسيجي لCathepsin D باستخدام تقنية المصفوفة الدقيقة للنسيج (TissueMicroarray) لمرضى سرطان القولون والمستقيم،واختبار التعبير الكيميائي المناعي النسيجي (Immunohistochemistry). حيث جمعت 110 من مرضى مصابين باورام القولون والمستقيم ، وكانت 90 من هذه العينات تعود الى مرضى مصابين باورام خبيثة و 20 منها لمرضى باورام حميدة ، فضلا عن مجموعة مكونة من 20عينة لمصابين بالتهاب الامعاء non specific colitis ، واخرى متكونة ايضا من 20 عينه ممن لايبدوعليهم انهم يعانون من اي امراض او اضطرابات معوية cases without significant pathology. تم الحصول على ثلاث قوالب من شمع البارافين تحتوي على جميع عينات الدراسة وذلك باستخدام تقنية المصفوفة الدقيقة للنسيج كما تم ادخال المعلومات الخاصة بتلك النماذج باستخدام برنامج الاكسل (Excel Microsoft) للحصول على خريطة مصفوفة دقيقة للنسيج (Tissue Microarray Map). اظهرت نتائج الدراسة وجود نسبة عالية من التعبير الكيميائي المناعي النسجيي

*Email:banjasim1975@yahoo.com

الموجب لCathepsin D في كل من مجموعتي الورم الخبيث(96.7%) والورم الحميد(80%) في حين كانت نسبة التعبير في مجموعة الالتهاب المعوي (30%).كما وجدت فروق معنوية(P<0.001)في التعبير الكيمياءي لCathepsin D بين انواع الورم الخبيث (adenocarcinoma and mucinous carcinoma) ومراحل الورم Tumor Stagesودرجات الورم Tumor Grades.

Introduction:

Among the various types of cancers, colorectal cancer (CRC) is a serious and common health problem worldwide [1]. Despite advances in surgical techniques and therapeutic interventions during the past few decades, CRC remains a major health problem worldwide due to therapy resistance [2]. Through more biological knowledge of tumorigenesis in CRC, more emphasis on early detection and development of new and improved treatment regimens [3]. A lot of research has been focused on the discovery and development of biomarkers to improve the diagnostic process and to predict treatment outcomes. Up till now only a few biomarkers are recommended by expert panels [4]. Discovery of additional prognostic markers might permit the development of guidelines for better management of CRC in order to improve overall survival. Although many biomarkers have been described, only a select few have provided prognostic data. Among those markers, Cathepsin D(CD); an aspartic lysosomal endopeptidase present in most mammalian cells, and is essential for regulating cell growth and tissue homeostasis of colon epithelium [5]. Overexpression of this protease has been associated with the progression of several human cancers [6]. During cancer progression, cathepsins are often translocated to the cell surface of tumor cells or are secreted into the extracellular environment, where they can promote tumor invasion through several possible mechanisms[7]. The role of CD in cancer has been postulated to promote tumor growth directly by acting to degrade and remodel the basement membrane and interstitial stroma surrounding the primary tumor and indirectly by stimulation of other enzymes or in cooperation with other cathepsins in the proteolysis process [8]. CD levels in tumors were reported to be higher than in adjacent normal tissue [9]. CD expression was suggested as an independent prognostic factor for poorer CRC specific survival [10]. The aim of this study is to assess the expression of CD in colorectal tissue samples, inflammatory and neoplastic, from Iraqi patients using TMA-IHC technique, and its relation with various clinicopathologic variables. This biomarker can aid with other standards in screening for disease, predicting progression, risk stratification and therapeutic benefit. This study was designed to assess the expression of Cathepsin D in colorectal tissue samples, inflammatory and neoplastic, from Iraqi patients, by Immunohistochemistry technique and correlation the expression of this marker with different clinicopathological variables as well as application of Tissue microarrays (TMAs) technique for assessment of immunohistochemical expression for this markers as this technique is newly applied on Iraqi studies.

Materials & Methods:

A total of 150 cases of colonic biopsies were collected from Iraqi patients during the period from January2014 to October 2014. Among these, 90 cases of colorectal cancer, 20 cases with benign lesions, 20 cases non-specific colitis and 20 cases reveal no significant pathology. Clinical information regarding patient's age, tumor size, grade, and pathological stage was obtained from the available histological reports. Hematoxylin and Eosin (H&E) stained sections were re-examined by two pathologists. All the preparations for tissue microarray (TMA) and immunohistochemistry (IHC) were performed in Pathology Unit - Southern General Hospital (SGH), University of Glasgow, United Kingdom.

Construction of tissue microarrays (TMAs)

Tissue cores of 0.6 mm in size were obtained from three paraffin blocks in this cohort. Five tumor tissue cores (0.6mm in diameter) were taken from each paraffin block with Beecher automated tissue array (Beecher Instruments, Sun Prairie, Wisconsin, USA). The cores were placed in a new recipient paraffin block that ultimately contained 325 tissue cores. The information of all TMA cases was put in data sheet which called TMA map. This TMA map consists of a simple Excel sheet, which served as a guideline to blocks arrangement and sequence in which they arrayed. Thus the TMA map was contained the exact location for each core in TMAs slide or block and another map (TMA code map) contained the code number for each core to each block. TMA block was cut at a thickness of 5µm on a microtome cutter (Leica RM2235). Sections were then placed on Salinized coated slides, (DAKO, UK) and heated at 58°C for 24 hours to be ready for the next step; the immunohistochemical staining.

TMA- Immunohistochemistry(IHC):

IHC was applied on TMA sections according to general immunohistochemical protocol. Staining was carried out for cathepsinD. Sections were deparaffinized in xylene and rehydrated in decreasing concentration of ethanol (100%, 90%, 80%, 70%). Antigen was retrieved using citrate buffer (prepared by dissolving 2.1 gm of citric acid powder into 1 litre of dH2O, adjust pH to 6) for 10min in pressure cooker. Slides were incubated in peroxidase – blocking solution (Dako, ready- to- use) for 20 min. Non-specific binding of antibodies was blocked by the addition of 2.5% normal horse serum, from (ImmPRESS™, Vector, USA).

Primary antibodies were diluted (1:1000) using antibody diluant (ready-to-use, Code No.ab64211 Abcam, Cambridge, UK), and incubated for 1hour at room temperature. Rabbit monoclonal antibodies kit from (Abcam, Cambridge, UK) was used to detect primary antibodies. Secondary antibodies (Anti Rabbit. peroxidase , Cat. No. MP-7401, ImmPRESS™ Vector, USA) was applied to the slides and incubated for 30 min at room temperature in a humidified chamber. The colorimetric detection of reaction was achieved by the Diaminobenzidine (DAB) Peroxidase Substrate method. Then, sections were counterstained with haematoxylin, dehydrated, and mounted. Tissue microarray slides scanned by using of digital image scanning analysis computer (NDP, U10074-01, UK).

Scoring system of IHC in TMAs:

Cathepsin D was shown in the cytoplasm of tumor cells, the scoring of this marker was done taking into consideration the proportion of positive cells (scored on a scale of 0-3) and staining intensity (scored on a scale of 0-3) [2]. Every tumor was given a score which represents the outcome of the summation of the intensity of the staining (intensity of score IS) (no staining = 0; weak = 1; intermediate staining = 2; strong staining = 3) with the percentage of stained cells (proportion score PS) (0% = 0); (1-25% =1); (26-50% = 2); (51-100%=3). The proportion and intensity were then summed to produce total scores of 0 or 1 through 6. A score of 0 was regarded as negative while 1- 2 as weak, 3-4 Moderate, 5 – 6 strong.

Statistical analyses

Statistical analysis was carried out using (SPSS V. 20). The association between cathepsin D and patient clinico-pathological features was assessed by chi -square test and Fisher exact test when the Chi square test was not fit. Statistical tests were approved by assuming a null hypothesis of no difference between variables, a probability was considered statistically significant at $P \leq 0.05$.

Results and Discussion:

The expression of CD was detected as a brown staining in the cytoplasm of tumor cells. All malignant cases showed positive expression of CD, it was moderate to strong in 96.7%, while 3.3% were weak. Regarding the adenoma cases, figures 1 and 3 showed strong expression of CathepsinD in 80% of those cases, and moderate expression in 20%, (Figures-1and 3).

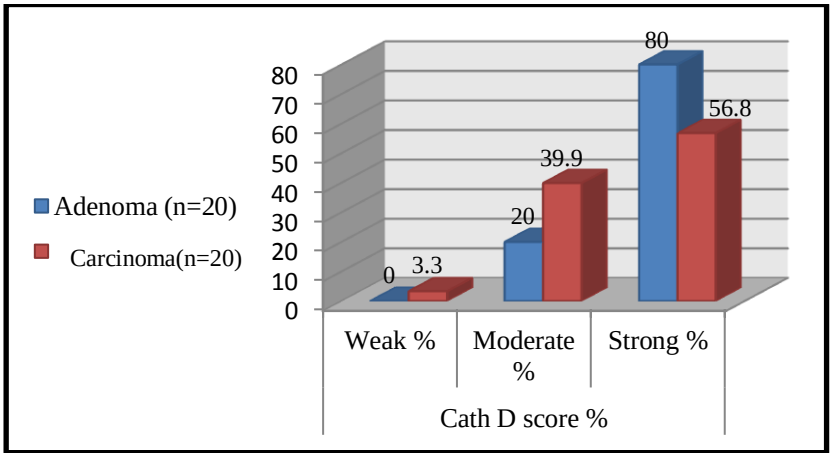

Figure 1-Percentage and scoring of immunohistochemical expression of Cathepsin D in colonic adenoma and colorectal carcinoma

Only 30% of colitis sections showed strong expression, in figures 2 and 3. Colonic biopsies with no significant pathology revealed weak to moderate expression with no strong expression, (Figures-2 and 3).

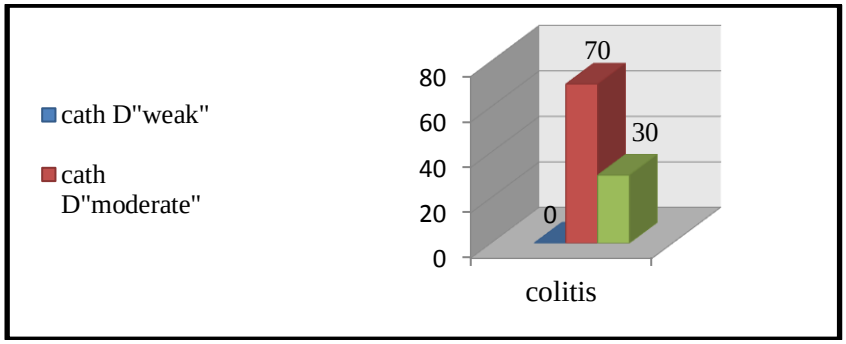

Figure 2- Percentage and scoring of immunohistochemical expression of Cathepsin D in colitis cases

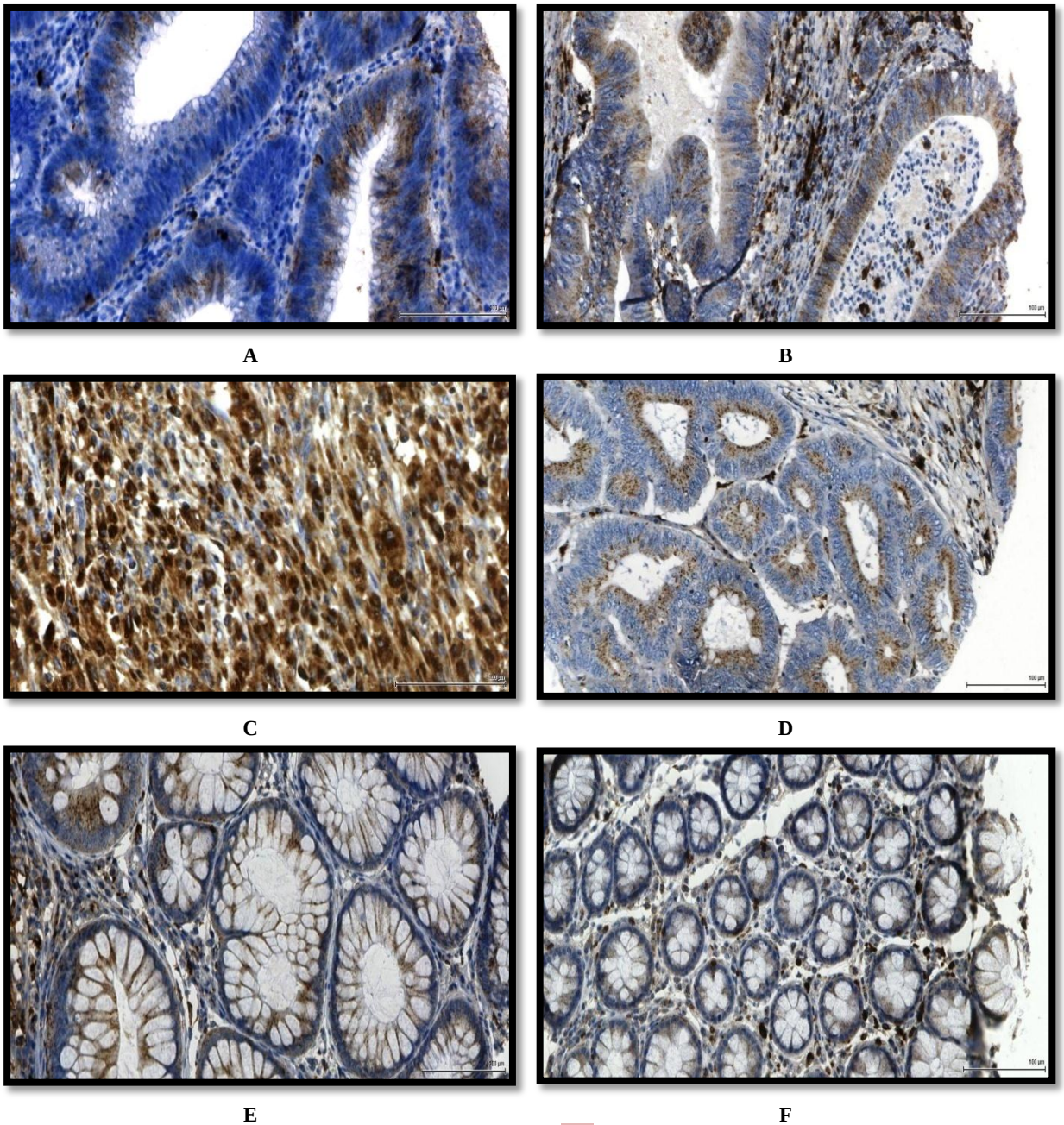

Figure 3- Immunohistochemical assessment of CathepsinD A:weak cytoplasmic expression of CathepsinD in moderately differentiated AC,(Hematoxylin& DAB, X 20)B: moderate cytoplasmic expression of CathepsinD in moderately differentiated AC,(Hematoxylin& DAB, X20) C: strong cytoplasmic expression of CathepsinD in poorly differentiated AC,(Hematoxylin& DAB, X 20) D:strong cytoplasmic expression of CathepsinD in tubular adenoma,(Hematoxylin& DAB, X 20)E:colitis

showing moderate cytoplasmic expresion of CathepsinD,(Hematoxylin& DAB, , X 20) F: normal colon tissue showing weak cytoplasmic expression of CathepsinD,(Hematoxylin& DAB, X 20).

Association of Cathepsin D expression with clinicopathological features

A- Association of Cathepsin D expression with age:

The results showed that no significant correlation between CD expression and the age in all groups, in Table-1.

Table 1- Association of CathepsinD expression with the age

Groups	Age(years)	No. of cases		Weak		Moderate		Strong		Fisher exact test	* P value
		n	%	n	%	n	%	n	%		
Carcinoma	**≤ 55**	43	47.7	1	1.1	13	14.4	29	32.2	2.5	0.378
	> 55	47	52.3	2	2.2	23	25.6	22	24.5		
Adenoma	**≤ 55**	14	70	0	0	4	20	10	50	0.2	0.267
	> 55	6	30	0	0	0	0	6	30		
Colitis	**≤ 55**	16	80	0	0	10	50	6	30	0.2	0.267
	> 55	4	20	0	0	4	20	0	0		
No significant pathology	**≤ 55**	17	85	8	40	9	45	0	0	0.4	1.00
	> 55	3	15	2	10	1	5	0	0		

(*****P< 0.05)

B- Association of Cathepsin D expression with gender:

Table-2 showed no significant differences in CD expression between male and female in the studied groups.

Table 2- Association of CathepsinD expression with gender

Groups	Gender	No. of cases		Weak		Moderate		Strong		Fisher exact test	*P value
		n	%	n	%	n	%	n	%		
Carcinoma	Male	53	58.8	2	2.2	26	28.8	25	27.8	7.5	0.076
	Female	37	41.2	1	1.1	10	11.3	26	28.8		
Adenoma	Male	18	90	---	---	4	20	14	70	0.6	1.00
	Female	2	10	---	---	---	---	2	10		
Colitis	Male	12	60	---	---	9	45	3	15	0.3	0.642
	Female	8	40	---	---	5	25	3	15		
No significant pathology	Male	14	70	7	35	7	35	---	---	0.3	0.642
	Female	6	30	4	20	2	10	---	---		

(*****P< 0.05)

C-Association of Cathepsin D expression with tumor site:

Table-3 showed no significant correlation between CD expression and location of tumor in adenoma and carcinoma groups.

Table 3- Association of Cathepsin D expression with tumor site

Groups	Site	No. of cases		Weak		Moderate		Strong		Fisher exact test	*P Value
		n	%	n	%	n	%	n	%		
Carcinoma	**Rt. Colon**	28	31.1	1	1.1	14	15.5	13	14.5	3.5	0.377
	Lt. Colon	62	68.9	2	2.2	22	24.4	38	42.3		
Adenoma	**Rt. Colon**	4	20	---	---	---	---	4	20	0.4	0.538
	Lt. Colon	16	80	---	---	4	20	12	60		

(*****P< 0.05)

D-Association of Cathepsin D expression with tumor type, stage & grade:

The present results showed that there were significant differences(P=0.023)in the expression of CD between adenocarcinoma & mucinous carcinoma, all mucinous cases showed strong expression in comparison with adenocarcinoma cases in which strong expressions was detected in 42 cases.

As well, results showed that there were high significant differences (P< 0.001) in the expression of CD in different stages and grades of tumor; that is, (34\36) of poorly differentiated cases showed strong expression of CD, compared to moderately differentiated cases in which, the strong expression was observed in (12\25) of cases and well differentiated cases in which only (5\29) of cases showed strong expression. Concerning tumor stage, the lowest proportion of strong expression of CD was observed in stage A (2\15) of cases, in comparison with stage C2 in which(19\20)of cases showed strong expression of this marker, Table-4.

Table 4- Association of Cathepsin D expression with tumor type, stage & grade

Carcinoma group		No. of cases		Weak		Moderate		Strong		Fisher exact test	*P value
		n	%	n	%	n	%	n	%		
Histological type	Adenocarcinoma	81	90	3	3.3	36	40.0	42	46.7	1.3	0.023 *
	Mucinous ca.	9	10	---	---	---	---	9	10		
Histological grade	Well diff.	29	32.3	2	2.2	22	24.6	5	5.5	3.3	< 0.001**
	Moderately diff.	25	27.7	1	1.1	12	13.3	12	13.3		
	Poorly diff.	36	40	---	---	2	2.2	34	37.8		
Dukes stage	A	15	16.6	1	1.1	12	13.3	2	2.2	2.9	< 0.001**
	B 1	15	16.6	1	1.1	8	8.9	6	6.6		
	B 2	19	21.1	1	1.1	8	8.9	10	11.1		
	C 1	21	23.3	---	---	6	6.6	15	16.7		
	C 2	20	22.2	---	---	1	1.1	19	21.1		

(*P< 0.05), (**P< 0.001)

Tumor invasion and metastasis is a highly complex phenomenon. Proteolytic enzymes have been suggested by several researchers as tumor markers in CRC [11], they are involved in the degradation of extracellular matrix, in colorectal cancer (CRC) invasion and metastasis, as well as in the malignant transformation of colorectal adenomas. Proteolytic enzymes may serve as potential target molecules for CRC therapy, and their use in combination with established chemotherapeutic strategies might have the potential to become a valuable oncological treatment form [12]. The metastatic process in cancer depends on the invasion of the tumor cells to the surrounding matrix and penetration of the basement membrane to reach the systemic circulation. These steps of degradation and membrane passage are used to be basically controlled by tumor associated proteolytic enzymes [13]. Of them, CD, a lysosomal aspartyl endopeptidase essential for regulating cell growth and tissue homeostasis of colon epithelium may be involved in CRC development and growth [14]. However, the prognostic value of CD overexpression in CRC is not well established as well as the correlation between CD expression in CRC and clinicopathlogical factors is still controversial [2]. Some authors have described a significant relationship between overexpression of CD and a trend towards advanced tumor stages, other studies have demonstrated the opposite [15].

Our examination of CD expression in TMAs from 90 patients with CRC by immunohistochemistry, suggested a high frequency of CD-positive tumor cells, actually, positive CD expression could be detected in all tumors of this study, it distributed as 56.8% strong expression, 39.9% moderate and 3.3% weak. This agree with previous study [16], who detected a high positivity rate (87.7%) based on immunohistochemical detection of CD, as well as another study[13] found that CD expression was positive in 91% of tumor cells. While other research reported that 82% of tumor cells were CD- positive expression[2]. In contrast, the recent study [17]observed a lower frequency (38.7%) of CD positive tumors.

The increased expression of CD may promote cancer cell proliferation or invasion and also associated with poor prognosis in cancer patients [10]. CD has been postulated to be secreted from cancer cells and been shown to serve as an autocrine growth factor in several cancer studies conferring proinvasive and prometastatic properties and also it plays a crucial paracrine role in the tumor micro-

environment by stimulating fibroblast outgrowth and tumor angiogenesis[18], this is because of increasing of invasive potential of the tumor cells due to CD production, thus increasing the probability of metastasis and to activate other proteases, which are also thought to be correlated with tumor progression [19]. Liaudet-Coopman *et al.* in 2006 concluded that CD over-expression stimulates tumorigenicity and metastasis, explaining that the CD is a key mediator of induced-apoptosis and its proteolytic activity has been involved generally in this event in which, mature lysosomal CD is translocated to the cytosol [20].

Alternatively, there were 56.8% of cases with strong expression of CD in tumor cells compared with cases of no significant pathology in which only weak and moderate expression of this marker had observed as recommended by previous studies[21- 22].The low expression of CD in tumor free tissues may be return to the fact that in normal cells, CD is localized in intracellular vesicles (lysosomes and endosomes) in comparison with cancer cells in which, overexpressed CD accumulates in cells, where it may affect their degradative capacities[21]. Conversely, the expression of CD in colitis biopsies and colonic adenoma, which is known to associate with the increase risk of colorectal carcinoma was lower than that of tumor cells, but it was higher than the expression in cases of no significant pathology as proved in previous studies [23, 24-25].On the other hand, a statistically significant relationship was detected between CD expression with the stage, grade and type of tumor. Importantly, in this study, CD expression is directly proportional to severity of disease and is highly elevated in late stages of CRC compared to earlier stages. Concerning the type of tumor, a significant relationship was observed between CD expression in the tumor cells and type of tumor (P=0.023), that is, all mucinous carcinoma cases, which represent the aggressive pattern of CRC, showed strong expression of CD in comparison with (42\81 of cases) of adenocarcinoma in which most of the strong expression was seen in poorly differentiated cases and stage C2 which represent late and worse stage in adenocarcinoma. This result match with Ibrahim *et al.* in 2012 who found that 71.4% of mucinous adenocarcinomas and 75% of signet ring cell carcinomas showed more intense CD expression (score+3) compared to 48.7% of conventional adenocarcinomas[13]. However, our results disagree with Famulski, *et al.* in 2001 who reported no significant relationship between different histopathological types of CRC and CD expression in tumor cells [28]. In respect to the grade of differentiation, a highly significant relationship was seen in this study between CD expression in the tumor cells and the grade of differentiation ($P< 0.001$), as poorly differentiated CRC showed the highest score (strong expression) of CD expression in (34\36 of cases). This result is consistent with Ibrahim *et al.* in 2012 who stated that poorly differentiated CRC showed the highest score of CD immunoreactivity (66.7% were score +3)[13], and Oh-e *et al.* in 2001 who agreed that poorly differentiated CRC showed the highest score of CD immunoreactivity[29]. However, this result was against the results reported by Ya jun *et al.* in 2006 and Kun lun *et al.* in 2010 who found no significant relationship between CD expression and grade of differentiation [27-30]. In this study, CD immunostaining was detected in high percentage of cancer cells of colorectal carcinomas and there was a significant increase in the expression in tumor cells of poorly differentiated carcinomas supporting the hypothesis that this marker may facilitate invasion of the surrounding stroma and predict high grade colorectal carcinomas.

Otherwise in this work, there was a shift toward higher scores of CD expression with advanced Dukes' stage, since 2.2%, 17.7% and 37.8% of Dukes A, B and C respectively showed highest score (+3) of CD expression, suggesting that CD expression might be associated with tumor progression. Some previous studies have described a significant relationship between overexpression of CD and a trend towards advanced tumor stage [13, 16-24].Our results also come in harmony with results reported by previous studies [27-31]who noted that expression of CD was highly elevated in late-stage CRC patients (TNM stage IV) compared to the earlier stages (TNM stages I, II, and III).Several studies have reported a wide range of CD and their antigen expressions patterns in colorectal tumors with the development of the disease stage, suggesting that CD may serve as a prognostic tumor marker and might be target for future therapy [32]. In this study, the association between the expression of CD with poor prognosis, demonstrates its potential as an independent prognostic factor of CRC in this population, and may be helpful in identifying patients with both late stage and potentially poor prognosis, which will help to determine if adjuvant chemotherapy is required or not to the patient.

References:

1. Bray, F., Ren, J., Masuyer, E. and Ferlay, J. **2013.** Estimates of global cancer prevalence for 27 sites in the adult population in 2008. *Int. J. Cancer*, 132(5):1133-1145.
2. Kirana, C., Shi, H., Laing,E., Kylie, H., Miller, R., Peter, B., Keating, J., Jordan,W., Hayes, M. and Stubbs, R**. 2012**. Cathepsin D expression in colorectal cancer: from proteomic discovery through validation using western blotting, immunohistochemistry, and tissue microarrays. *Int. J. Proteomics*, article ID 245819, 10 pages.
3. Jemal, A., Bray, F., Center, M., Ferlay, J., Ward, E. and Forman, D. **2011**. Global cancer statistics. *Ca. Cancer J. Clin.*, 61(2):69-90.
4. Marlies, S., Reimers, E., Peter J., Gerrit, J. and Cornelis J. **2013**. Biomarkers in precision therapy in colorectal cancer. *Gastroenter. Rep.*, 1(3):166-183.
5. Tejpar, S., Bertagnolli, M., Bosman, F., Lenz, H., Garraway, L., Waldman, F., Warren, R., Bild, A., Collins, D., Hahn, H., Kennedy, R., Ilyas, M., Morreau, H., Proutski, V., Swanton, C., Tomlinson, I., Delorenzi, M., Van, E. and Roth A. **2010**. Prognostic and predictive biomarkers in resected colon cancer: current status and future perspectives for integrating genomics into biomarker discovery. *Oncologist*, 15:390–404.
6. Jacobson-Raber, G., Lazarev, I., Novack, V., Mermershitein, W., Yael, B., Geffen, D., Vardy, N. and Ariad, S. **2011**. The prognostic importance of cathepsin D and E-cadherin in early breast cancer: a single-institution experience, *Onco. Lett.*, 2(6):1183–1190.
7. Shi, H., Hood, K., Hayes, M. and Stubbs, R. **2011**. Proteomic analysis of advanced colorectal cancer by laser capture microdissection and two-dimensional difference gel electrophoresis. *J of Proteomics*, 75(2):339–351.
8. Derocq, D., Prébois, C., Beaujouin, M., Laurent-Matha, V., Pattingre, S., Smith, G. and Liaudet-Coopman, E. **2012**. Cathepsin D is partly endocytosed by the LRP1 receptor and inhibits LRP1-regulated intramembrane proteolysis. *Oncogene*. 31(26):3202-3212.
9. Szajda,S., Snarska, J., Jankowska, A., Roszkowska-Jakimiec,W., Puchalski, Z. and Zwierz, K. **2008**. Cathepsin D and carcino-embryonic antigen in serum, urine and tissues of colon adenocarcinoma patients, *Hepatogastroenterology*, 55(82-83):388–393.
10. Shin, I., Sung, N., Lee, Y., Kwon , T., Si, Y., Lee, Y., Oh, S. and Lee, I. **2014**. The expression of multiple proteins as prognostic factors in colorectal cancer: cathepsin D, p53, COX-2, epidermal growth factor receptor, C-erbB-2, and Ki-67.*Gut. Liver*, 8(1):13-23.
11. Anestakis,D., Argyraki,M., Petanidis, S. and Iakovidou-Kritsi, Z. **2013**.Assessment of the roles of cathepsins B, H and L in the progression of colorectal cancer. *J. of can. ther.*, 4: 1-7.
12.Ényi, L.,Barabás, L., Hritz, I., István, G. and Tulassay, Z. **2014**. Impact of proteolytic enzymes in colorectal cancer development and progression. *W. J. Gastroenter.* 20(37): 13246-13257.
13. Ibrahim, B., Helmy, D., El.Sheikh, S. and Mostafa, R. **2015.** Immunohistochemical expression of galectin-3 in colorectal carcinoma *Mid. Eas. J. of Sci. Res.*, 23 (4): 580-591.
14. Sis, B., Sagol, O., Ku, P., Sokmen, S., Terzi, C., Fuzun, M., Ozer, E. and Bishop, P. **2004**. Prognostic significance of matrix metalloproteinase-2, cathepsin D, and tenascin-C expression in colorectal carcinoma, *Pathol. Res. Pract.*, 200 (5): 379-387.
15. Kuester, D., Lippert, H., Roessner, A. and Krue-Ger, S.**2008**. The cathepsin family and their role in colorectal cancer. *Path. Res. and Prac.*, 204: 491-500.
16. Mayer, A., Fritz, E., Fortelny, R., Kofler, K. and Ludwig, H.**1997**. Immunohistochemical evaluation of cathepsin D expression in colorectal cancer. *Can. Invest.*, 15(2):106-110.
17. Theodoropoulos, G., Panoussopoulos, D., Lazaris, A. and Golematis, B. **1997**. Evaluation of cathepsin D immunostaining in colorectal adenocarcinoma, *J. of Surg. Onco.* , 65(4): 242–248.
18. Benes, P., Vetvicka, V. and Fusek, M. **2008**. Cathepsin D many functions of one aspartic protease.critical review in oncology. *Hematology*, 68(1): 12–28.
19. Abbott, D., Margaryan, N.,Jeruss, J., Khan, S., Kaklamani, V., Winchester, D.,Hansen, N., Rademake, A., Khalkhali-Ellis, Z. and Hen-Drix, M. **2010**. Reevaluating cathepsin D as a biomarker for breast cancer serum activity levels versus histopathology. *Can. Biol. Ther.*, 9 (1): 23-30.
20. Liaudet-Coopman, E., Beaujouin, M., Derocq, D., Garcia, M., Glondu-Lassis, M., Laurent-Matha, V., Prébois, C., Rochefort, H. and Vignon, F. **2006**. Cathepsin D: newly discovered

functions of a long-standing aspartic protease in cancer and apoptosis. *Cancer Lett.*, 237:167–179.

21. Iacobuzio-Donahue,C.,Shuja,S., Jinguo,C., Phyllis, P., Willett, J. and Jo M. **2004**. Cathepsin D protein levels in colorectal tumors: divergent expression patterns suggest complex regulation and function. *Int. J. Oncol.*, 24(3):473-485.

22. Masson, O., Bach, A., Derocq, D., Prébois, C., Laurent-Matha, V., Pattingre, S. and Liaudet-Coopman, E. **2010**. Pathophysiological functions of cathepsin D: targeting its catalytic activity versus its protein binding activity? *Biochimie.*, 92(11):1635-1643.

23. Galandiuk, S., Miseljic, S., Yang, A., Early, M., McCoy, M. and Wittliff, J. **1993**. Expression of hormone receptors, cathepsin D, and HER-2/neu oncoprotein in normal colon and colonic disease, *Arch. of Surg.*, 128(6):637–642.

24. Talieri, M., Papadopoulou, S., Scorilas, A., Xynopoulos, D., Arnogianaki, N., Plataniotis, G., Yotis, J. and Agnanti, N. **2004**. Cathepsin B and cathepsin D expression in the progression of colorectal adenoma to carcinoma. *Cancer Lett.*, 205(1):97-106.

25. Kunimura,T., Tomoko, I., Wada, M., Ushio, J., Sato, K., Tetsuji, E., Masanao, N., Hirotaka, K., Ryouji, H., Saitou, K. and Morohoshi, T.**2003**. Immunohistochemical evaluation of tissue-specific proteolytic enzymes in adenomas containing foci of early carcinoma. *Int. J.of Gastro. Ca*., 33(2-3):149-154.

26. Hausmann, M., Obermeier, F., Schreter, K., Spottl, T., Falk, W., Scholmerich, J., Herfarth, H., Saftig, P. and Rogler, G.**2004**. Cathepsin D is up-regulated in inflammatory bowel disease macrophages. *Clin. Exp Immunol.*, 136(1):157-167.

27. Kun-Lun, Y., Zhi-Jian, D., Hui-Jie, H. and Yong, L. **2010**. Expression of cathepsin D in colorectal cancer. *Inner. Mon. med. J.*, DOI: CNKI: SUN: NMYZ.0. -01-007.

28. Famulski, W., Guzińska-Ustymowicz, K., Sulkowska, M., Chabowski, A., Zalewski, B., Piotrowski, Z., Stasiak-Barmuta, A. and Sulkowski, S. **2001**. Tumor budding intensity in relation to cathepsin D expression and some clinicopathological features of colorectal cancer. *Folia Histochem. Cytobio.*, 39 (2): 171–172.

29. Oh-e, H., Tanaka, S., Kitadai, Y.,Shimamoto, F., Yoshihara, M. and Haruma, K. **2001**. Cathepsin D expression as a possible predictor of lymph node metastasis in submucosal colorectal cancer. *Eur. J. of Cancer*, 37(2): 180–188.

30. Ya-Jun, T., Dong-Qing, W., Jie, Y. and Liaoning, Z. **2006**. Mucins-2 and cathepsin D expressions in colorectal cancer provincial key laboratory of bioorganic chemistry. *Chin. J. of clin. Gastroenter*,1.

31. Sebzda, T., Saleh, Y., Gburek, J., Andrzejak, R., Gnus, J., Siewinski, M. and Grzebieniak, Z. **2005**.Cathepsin D expression in human colorectal cancer: relationship with tumour type and tissue differentiation grade. *J. Exp. Ther. Oncol*., 5 (2): 145-150.

32. Grill, S., Brown, C. and Miller, R. **2011**. Colon Cancer. a practical Guide. *Springer*, pp: 337.