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Running Title: Leptin and Cyclin D1 in Colon Cancer
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Evaluation of the Prognostic Role of Leptin and Cyclin D1 Expression in Colorectal Cancer

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Abstract

Background: Colorectal cancer (CRC) is one of the most malignancies worldwide. This study aimed to determine the role of leptin and cyclin D1 in CRC and their association with clinicopathological parameters and clinical outcomes of patients, and to compare them with normal tissues.

Method: A retrospective study was carried out on 60 specimens classified as 8 normal colonic mucosae, 40 patients suspected to have CRC, and 12 colonic dysplasia (CD) and were evaluated in terms of tumor node metastases staging using multislice computerized tomography scan, quantitative real-time polymerase chain reaction to calculate relative leptin mRNA expression level; and immunohistochemical staining was performed to study leptin and cyclin D1 expressions in CRC and CD versus the normal colonic mucosa. Normal mucosa was obtained by colonoscopy from patients for causes other than neoplasia or dysplasia. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 24 Inc. Chicago, IL, USA) with a significance value of $P \leq 0.05$.

Results: There was a significant association between leptin and cyclin D1 positive expressions and both CRC and CD. However, poor clinical outcome was significantly correlated with high positive leptin and cyclin D1 expressions detected in CRC patients. Cyclin D1 and leptin showed a strong association with greater histological grade, stage, positive LN, and distant metastases of CRC.
Conclusion: Expressions of leptin and cyclin D1 are associated with colon disorders with unfavorable outcomes in colon cancer. New opportunities may emerge from the discovery of cross-talk between leptin and other oncogenic pathways in obesity-related cancers, with a particular focus on colon cancer.

***Keywords*:** Leptin, Cyclin D1, Obesity, Colorectal disorders

Introduction

Colorectal cancer (CRC) is the most common malignant tumor of the gastrointestinal tract. It is the third most common type of cancer in males and the second most common type of cancer in females. Moreover, it represents the fourth most common cause of death due to cancer in the world.[1] Due to their subtle nature, a high percentage of early CRC findings are missed on abdominal computerized tomography (CT), with the majority of misses occurring in the rectosigmoid and ascending colon. Early detection by specifically looking for polyps, wall thickening, and small lymph nodes can be difficult to diagnose. The early detection of CRC could improve survival by lowering the stage from 3C to 3A, resulting in a 36% increase in 5-year survival.[2] Moreover, the extramural invasion has a significant impact on the prognosis of patients with CRC.[3] Unfortunately, CRC is resistant to chemotherapy and tends to recur and spread. Moreover, several treatment protocols have been applied to CRC, but they have not yielded a complete cure. This may be because CRC stem cells are resistant to radiotherapy and chemotherapy and are able to proliferate.[4] Therefore, identifying factors that may influence CRC aggressiveness and allow a more accurate diagnosis as well as prognosis is absolutely essential.[5]

CRC occurs in different populations either sporadically (75-80%) or hereditary (20-25%). There are many predisposing factors for CRC including previous presence of polyps (adenomas), previous presence of ulcerative colitis, inflammatory bowel diseases, hereditary familial adenomatous polyposis, and nutritional factors such as low-fiber diet.[5] Obesity and/or adipose tissue endocrine disruption are suggested to be related to cancer development and prognosis, but the underlying mechanisms are unclear.[6] Leptin is a 167-amino-acid peptide hormone secreted primarily by adipocytes and encoded by the ob (obese) gene located on chromosome 7. The main function of leptin is to regulate body mass through negative feedback between adipose tissue and the satiety center in the hypothalamus. It is involved in stimulating energy consumption and reducing appetite.[7] The anti-apoptotic and mitogenic effects of leptin on various cancer cell lines as well as promoting cell growth, migration and invasion have been documented.[8] Furthermore, there is some evidence suggesting a potential role for leptin as a novel growth factor driving the development and progression of CRC, but large clinical studies are needed to determine the mechanisms underlying the effects of leptin on the development and progression of this cancer.[9] Leptin plays a critical role in the cell cycle by increasing the S-phase cells, along with increasing cyclin D1.[10]

Cyclin D1 is important for the cell cycle because it regulates the progression from the G_1 phase of the cell cycle to the S phase which is mediated by its interactions with cyclin-dependent kinases 2, 4 and 6.

The action of cyclin D1 can be blocked by cyclin D1-dependent kinase inhibitors, such as p27 and p21.[11] Increased expression of cyclin D1 has been documented in a variety of tumor types, so greater focus has been placed on its involvement in tumor development and progression.[12] Overexpression of cyclin D1 has been shown to be associated with worse clinicopathological signs and prognosis of estrogen receptor (ER)-positive breast cancer as well as other cancers.[13] Some authors have evaluated whether overexpression of cyclin D1 may be a prognostic factor for survival in patients with CRC. However, the results are inconclusive, and no consensus has been reached.[12] Therefore, this work aimed to study leptin and cyclin D1 expressions in CRC and colonic dysplasia (CD) versus normal colonic mucosa.

Material and Methods

Patients and study design

A retrospective study was performed including CRC cases as well as normal colon mucosa samples as a control group in the Departments of Pathology, Surgery, Internal Medicine, Tropical Medicine, Diagnostic Radiology, Clinical Biochemistry and Molecular Biology of Faculty of Medicine from June 2022 to June 2023. All patients provided full written consent to join the study with no identity details. The study was approved by IRB-committee, Faculty of Medicine, (IRB approval number ZU-IRB #6993/2-10-2022) and was performed following the ethical standards of the Helsinki Declaration.

Patients included in this study complained of one or more of the following symptoms: iron deficiency anemia (IDA), rectal bleeding, bowel habits changes, weight loss, bowel obstruction/perforation, and abdominal mass. Pregnant and lactating women, patients diagnosed with types of cancer other than CRC, and patients receiving chemotherapy or radiotherapy were excluded from this study.

All patients underwent complete history taking, complete physical examination, digital rectal examination, multislice CT evaluation, and TNM staging of CRC cases according to AJCC/2017, where T stands for tumor evaluation size and appearance, N stands for nearby abnormal lymph nodes having cancer, and M stands for mesenteric infiltrate and distant metastasis.[13] Subsequently, partial or total colectomy was performed in cases of colon cancer, and colonic polypectomy in cases of colonic adenoma and CD. Tumor samples as well as normal colon tissues from some cases of colposcopies were then obtained and stored at −80°C until further processing. Finally, the tissues were processed for routine histological examinations using hematoxylin and eosin (H&E) staining.[15] In addition, the following investigations were conducted.

The specimens were classified into 3 groups as follows: group 1 (G1) containing normal colonic mucosae, group 2 (G2) formed of cases of CD polyps and group 3 (G3) containing CRC. G1 samples were obtained from patients who underwent colonoscopy for reasons other than neoplasia, such as ulcerative colitis and irritable bowel syndrome, and were used as a control group.

RNA extraction and quantitative real-time polymerase chain reaction (qPCR) analysis

Specimens of tissue samples were homogenized, followed by extraction of total cellular RNA from tissue homogenate using easy-RED™ Total RNA extraction kit (iNtRON Biotechnology, Seongnam, Korea) following the manufacturer's instructions. Reverse transcription of one µg of RNA was performed by means of Maxime RT PreMix Kit (iNtRON Biotechnology, Seongnam, Korea) according to the manufacturer's protocol. qPCR was performed as follows: 10 µl of TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX), 1 µl of each forward & reverse primers, 2 µl of cDNA and 6 µl of RNAase free water in 20 µl final volume.

GAPDH gene expression was used as internal control. 40 cycles; initial denaturation at 95°c for 15 minutes, followed by 95°c for 30 seconds, 53°c for 1 minute and 72°c for 1 minute. The thermal program for the replication of the three genes done under the same conditions containing, the sequences of leptin qPCR primers were forward primer, 5′-ACAGAAAGTCACCGGTTTGG-3′; reverse primer, 5′-GCTCTTAGAGAAGGCCAGCA-3′, for GAPDH forward: 5'-ATGGAGAAGGCTGGG GCT-3' and GAPDH reverse: 3'-ATCTTGAGGCTG TTGTCATACTTCTC-5'. Leptin relative mRNA expression levels was normalized to GAPDH as housekeeping gene. The relative fold changes in mRNA expression levels were calculated and determined using the threshold cycle (CT) method (2-ΔΔCT method).[16]

Immunohistochemical staining (IHC)

Four-micron sections of each tissue specimen were cut onto positive-charged slides; air dried overnight, deparaffinized in xylene, hydrated through a series of graded alcohol and washed in distilled water and 0.01 PBS (pH 7.4). Slides were then processed for IHC as described by Handa et al. using the following antibodies: leptin (MIB-1, Dako), and cyclin D1 (DCS-6, Dako).[16] A case of mantle cell lymphoma was used as a control for cyclin D1. Negative controls were obtained by replacing the primary antibody by non-immunized rabbit or mouse serum.

IHC analysis

Assessment of leptin immunohistochemistry staining was performed by calculating the frequency of positive cells by applying a semiquantitative method. Staining intensity has been given scores 0, 1, 2, and 3 demonstrating negative, mild, moderate, and strong staining, respectively.[17] Assessment of cyclin D1 IHC staining was reported as negative, when positive brown nuclear stained cells less than 5%; mild, when positive brown nuclear stained cells 5–25%; moderate, when positive brown nuclear stained cells 26–50%; strong, when positive brown nuclear stained cells >51%. No cytoplasmic staining of cyclin D1 was found in any of the studied cases. Scores of both markers have been grouped as negative, low (1+) and high (2+ and 3+), as was reported previously.[17,18] All interpretations of IHC have been performed by 3 pathologists.

Statistical analysis

The collected data were computerized and statistically analyzed using Statistical Package for Social Sciences (SPSS 24 Inc. Chicago, IL, USA). Data were tested for normal distribution using the Shapiro Walk test. Qualitative data were represented as frequencies and relative percentages. Chi square test ($\chi 2$) was used to calculate difference between qualitative variables as indicated. Quantitative data were expressed as mean ± SD (Standard deviation). Independent T test was used to calculate difference between quantitative variables in two groups. One-way ANOVA F-test was used to calculate difference between quantitative variables in more than two groups with Post hoc test for multiple comparisons was done using LSD method. To compare the two groups, the Mann-Whitney test was used. The Kruskal-Wallis method was used to compare data that were not normally distributed in the three independent groups. All statistical comparisons were two tailed with significance of P-value ≤ 0.05, while $P < 0.001$ indicated a highly significant difference.[16,19,20]

Results

This prospective cross-sectional study included a total number (N) of 60 tissue specimens from 60 patients; 70% were male, while 30% were female. Also, 65% of the studied cases were obese with body mass index (BMI) more than 30, and the rest of them were normal. The number of cases in each group was as follows: G1: 8 (13.3%) control cases, G2: 12 (20%) CD and G3: 40 (66.7%) CRC. Most common

presentations in CRC patients were weight loss (25%), and IDA (25%), followed by bleeding per rectum (12.5%) and changes in bowel habits (12.5%) (Table 1). Some patients experienced more than one symptom.

Regarding gene expression levels, qPCR analyzes revealed that significantly higher expression levels of leptin and cyclin D1 mRNA were observed in CRC compared with CD, and in CD as compared with control tissues (Table 2; Figure 1A). High leptin and cyclin D1 mRNA expression were significantly associated with age group >50 years (P <0.0001) and obesity (P <0.0001). Nonetheless, there was no association between leptin and cyclin D1 mRNA expression and gender in the studied population (P = 0.09, 0.056, respectively) (Table 3).

Among the CRC patients, there was a significant positive relation between leptin and cyclin D1 with an association coefficient of 0.67 (P < 0.0001) (Figure 1B). With the investigation of the association of leptin mRNA expression with the clinicopathological criteria of the CRC patients, the expression in tissues was found to be significantly associated with tumor grades 3 & 4 (P = 0.018), staging 3 & 4 (P = 0.026), tumor size (P = 0.014), lymph node, and distant metastasis (P < 0.0001). The association of cyclin D1 mRNA expression with the clinicopathological data indicated that its overexpression was significantly associated with the pathological stages 3 and 4 (P = 0.043), LN metastasis (P = 0.005), and distant metastasis (P = 0.0001) of CRC; however, it showed no association with tumor grade (P = 0.14) and size (P = 0.06) (Table 4; Figure 2).

The IHC expressions of leptin and cyclin D1 markers were negative in 11.7% and 13.3%, and positive in 88.3% and 86.7% of the studied groups, respectively. Positive expression of leptin and cyclin D1 was low (mild) in 13.3% and 15.0%, and high in 75% and 71.6%, (including moderate in 20.0% and 33.3%; and strong in 55.5% and 38.3%) of the studied groups, respectively (Table 5; Figure 3).

Positive leptin IHC expression was significantly associated with age group (49 ± 10), obesity (69.8%), CD (20.8%) and CRC (75.5%) (P = 0.0460, 0.032, <0.001, respectively). Yet, there was no relationship between leptin expression and gender in the studied population (P = 0.334). Meanwhile, positive cyclin D1 IHC expression was significantly correlated with CD (21.2%) and CRC (76.9%), (P < 0.001). However, no significant relationship between cyclin D1 expression, age, gender and obesity in the studied groups, (P = 0.128, 0.246, 0.08, respectively), (Table 6). Significant co-expression of cyclin D1 and leptin was observed both negatively (87.5%) and positively (100 %), (P < 0.001), respectively (Table 7; Figure 3).

Significant strong positive expressions of both leptin and cyclin D1 IHC markers were observed in CRC cases with histological grade III (25% and 17.5%, respectively) & with grade IV (45%, and 32.5%, respectively), tumor size than > 5cm (60% and 50%, respectively), positive LN metastasis (62.5% and 50%, respectively), positive distant metastasis (45%, and 47.5%, respectively), stage III (25%, and 45%, respectively) and stage IV (7.5%, and 42.5 %, respectively) (Table 8; Figures 2 and 3).

Discussion

The present study investigated the association of leptin as well as cyclin D1 immunohistochemical markers expression in CD and CRC. Cyclin D1 and leptin showed marked expression in CRC and CD rather than in normal colon tissue. There was also a significant association between positive leptin expression and obesity. This shed the light on the role of leptin and its link to obesity in cases of colon dysplasia pathogenesis and its progression to colon cancer. Cyclin D1 and leptin are significantly correlated with higher

histological grade, stage, positive LN and distant metastasis.

CRC is one of the most common and aggressive malignancies worldwide. It represents the second leading cause of death due to cancer worldwide. Obesity, as well as other risk factors, may play a role in colon carcinogenesis.[21] Both obesity and CRC are associated with a sedentary lifestyle, consumption of high-energy diets, and low consumption of fruits and vegetables.[9] In the present study, there was a significant association between leptin expression and both age and obesity, with significant positive leptin expression mainly in older age and obese groups. However, there was no significant association between the gender of patients and leptin markers expression in the studied groups. Also, qRT-PCR results showed that the elevated leptin and cyclin D1 mRNA expression was significantly correlated with age >50 years and obesity. Nevertheless, there was no association between them and gender in the studied groups. Other studies found in accordance with our study that obesity has been shown to be a risk factor for the development of CRC, with higher leptin expression in CRC patients.[9,21,22] On the other hand, Li et al. reported that no significant expression relationship was found between leptin with age or with gender of CRC patients.[23]

Regarding cyclin D1 expression, our examination showed no significant association between it and age, gender and obesity in the studied groups. Al-Maghrabi et al. and Albasri et al. agreed that cyclin D1 overexpression did not reveal any significant correlation with age and gender.[17, 18] However, some authors have suggested that CRC is a hormone-dependent cancer and is more common in males than in females.[24] Li et al. also stated that cyclin D1 overexpression was associated with a higher proportion of older patients (60 years).[11] Jun et al. disagreed and revealed that cyclin D1 intensity of expression was significantly higher in female CRC patients and younger age groups.[25] Discrepancies in the results of these studies may be due to differences in human races, sample sizes, tissue sections, and cutoff points that can affect the results of prognostic evaluation of cyclin D1 expression.

The present results also showed that cyclin D1 expression is significantly higher in CRC cases followed by CD cases compared with normal cases. Li et al. agreed that cyclin D1 is overexpressed in many human cancers, including CRC, and plays an important role in cancer cell cycle progression.[11] In addition, Albasri et al. confirmed increased overexpression of cyclin D1 in the progression from normal tissue to adenoma to cancer suggesting a carcinogenic role.[18]

In this study, the immunohistochemical expression level of leptin and its association with CD and CRC has also been studied. It revealed a significantly higher expression of leptin in CRC cases compared with CD cases, and in CD compared with normal cases. These findings go hand in hand with that of Li et al. and Erkasap et al. who found that leptin and cyclin D1 mRNA expression is increased in CRC tissues compared with normal tissues suggesting a critical role of leptin in cancer development.[23,26] Koda et al. also found that immunoreactivity for leptin was detected in 51.2% (85/166) of primary CRC.[23] Al-Maghrabi et al. agreed that leptin has been found in a very high percentage (93%) of the samples of CRC on immunostaining.[27] Moreover, immunoreactivity for leptin was observed in 51.2% (85/166) of primary CRC. However, Perumal et al. reported that leptin was also expressed in other tumors such as renal cell carcinoma.[28] This could reinforce the specific role of leptin in the development of various types of cancer.[29]

Significant co-expression of cyclin D1 and leptin was observed in our study in both negative (87.5%) and positive (100%) responses ($P < 0.001$). This has been approved in study of human hepatocarcinoma by Chen et al. who suggested that cyclin D1 immunoreactivity

was enhanced in leptin-treated cells, as leptin up-regulates cyclin D1 expression to promote cell proliferation and down-regulates pro-apoptotic protein.[30] Our present results showed that there was strong and statistically significant positive co-expression of both leptin and cyclin D1 IHC markers in CRC cases with histological grades III and IV, tumor size >5 cm, positive LN metastasis, positive distant metastasis, and stages III and IV. Based on these results, it has been suggested that positive expression of both leptin and cyclin D1 IHC can be associated with poor prognosis in CRC.

Based on qRT-PCR results, leptin mRNA expression was significantly associated with tumor grades 3 and 4, stages 3 and 4, tumor size, lymph node, and distant metastases. The overexpression of cyclin D1 mRNA was significantly associated with the pathological stages 3 and 4, LN metastasis, and distant metastasis of colonic cancer; however, it showed no association with tumor grade and size. There was also a significant positive association between leptin and cyclin D1. This may indicate that the biological effect of leptin in regulating cancer cell proliferation may be mediated by an increase in cyclin D1 expression which is in line with what Lin and Hsiao observed.[31] Li et al. agreed that leptin overexpression is a poor prognostic factor for CRC.[23] Moreover, other studies have indicated that overexpression of cyclin D1 is a predictor of poor prognosis associated with poor clinical outcome in CRC patients.[11, 18, 32]

However, in disagreement with us, Al-Maghrabi et al. stated that cyclin D1 immunoexpression cannot be used as a predictor of survival in CRC.[17] It also does not show any significant association with clinicopathological features except for lymphovascular invasion. Moreover, Jun et al.pointed out that cyclin D1 expression can be used as a favorable prognostic indicator in patients with CRC.[25] Jeong et al. also reported high leptin expression level was found to be inversely associated with nodal stage.[33] They also added that a higher leptin expression level might also be a predictor of a better oncologic outcome. This may be due to aberrant signaling as well as leptin genetic variations associated with obesity and CRC. High expression, rare mutations, and single nucleotide polymorphisms have been found in obesity, leading to the identification of an association between leptin genetics, obesity, and colon cancer risk.[6] Other authors also revealed that leptin is correlated with better prognosis in CRC patients.[34,35] This might be due to leptin paradox which means better outcomes in CRC patients with high BMI by inhibiting tumor growth and promoting patients' survival during the disease depending on the amount of leptin stored in adipose tissue.

Prognostic biomarkers can significantly improve the choice of treatment protocol for CRC patients. Early detection of CD and its differentiation from neoplastic colon lesions enables surgeons to perform laparoscopic resection of focal dysplasia, rather than colectomy.[36] The risk of developing CRC increases with longer duration of colitis and greater anatomic extent of colitis. This also indicates that the pathogenesis of CRC in inflammatory bowel disease (IBD) follows a sequence of inflammation, dysplasia, and carcinogenesis.[36,37]

The present study may have some limitations. They may include a relatively small number of samples. Furthermore, this work evaluated leptin mRNA in general and its association with colon dysplasia as a predictive indicator, without taking into account different mutations. Furthermore, distinct leptin genetic variations, leptin receptor mutations, and polymorphisms may alter the prognosis of colon cancer. Therefore, BMI can also be examined to determine its relationship to obesity-related colon cancer. Studies of leptin association are limited to differences detected at the time of inquiry. Different ethnic backgrounds with multiple characteristics could also be studied and integrated in

future research, as our study was conducted in Egypt only.

Conclusion

Cyclin D1and leptin show marked expression in colon disorders with unfavorable outcomes in colon cancer. Therefore, it is suggested that both leptin and cyclin D1 can be used as markers of poor prognosis in CRC. It is recommended that further studies investigate larger numbers of patients, and in different ethnicities regarding the association of cyclin D1 and leptin in predicting CRC prognosis before adopting it in clinical practice.

Availability of Data and Materials

The datasets used and/or analyzed in the present study are available on reasonable request.

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Authors' Contributions

S.M.H: Study design, data gathering, drafting and reviewing the manuscript; A.A.H: Study design, data analysis, interpretation and reviewing the manuscript; F.E: Study design, data gathering, drafting and reviewing the manuscript; I.M.E: Data gathering, drafting, and reviewing the manuscript; A.E: Study design, reviewing the manuscript; M.A.Z: Study design, drafting and reviewing the manuscript; B.A.I: Study design, drafting and reviewing the manuscript; M.A.A: Data gathering, drafting, and reviewing the manuscript; H.M.A: Study design, drafting and reviewing the manuscript; M.M.E: Data gathering, drafting, and reviewing the manuscript; A.H.A: Data gathering, drafting and reviewing the manuscript; M.A.G: Study design, data gathering, drafting and reviewing the manuscript; All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Conflict of Interest

None declared.

References

1. Al-Shamsi HO, Jones J, Fahmawi Y, Dahbour I, Tabash A, Abdel-Wahab R, et al. Molecular spectrum of KRAS, NRAS, BRAF, PIK3CA, TP53, and APC somatic gene mutations in Arab patients with colorectal cancer: determination of frequency and distribution pattern. *J Gastrointest Oncol.* 2016;7(6):882-902. doi: 10.21037/jgo.2016.11.02. PMID: 28078112; PMCID: PMC5177568.
2. Rodriguez R, Perkins BD, Park PY, Koo P, Kundranda MN, Chang J. Early colorectal cancer detection on routine CT to improve patient's 5-year survival. *J Clin Oncol*. 2018;36(4_suppl):593. doi:10.1200/jco.2018.36.4_suppl.593.
3. Elibol FD, Obuz F, Sökmen S, Terzi C, Canda AE, Sağol Ö, et al. The role of multidetector CT in local staging and evaluation of retroperitoneal surgical margin involvement in colon cancer. *Diagn Interv Radiol.* 2016;22(1):5-12. doi: 10.5152/dir.2015.15089. PMID: 26611110; PMCID: PMC4712898.
4. Harb OA, Hegazy AA, Gertallah L, Haggag RM, Mostafa SH. Prognostic value of immunohistochemical expressions of the stem cell biomarker "LGR5"

and the proliferation biomarker "TPX2" in colorectal carcinoma. *J Tum*. 2016;4(3):426-34. doi:10.17554/j.issn.1819-6187.2016.04.93.
5. Roshandel G, Ghasemi-Kebria F, Malekzadeh R. Colorectal cancer: Epidemiology, risk factors, and prevention. *Cancers (Basel)*. 2024;16(8):1530. doi: 10.3390/cancers16081530. PMID: 38672612; PMCID: PMC11049480.
6. Socol CT, Chira A, Martinez-Sanchez MA, Nuñez-Sanchez MA, Maerescu CM, Mierlita D, et al. Leptin signaling in obesity and colorectal cancer. *Int J Mol Sci*. 2022;23(9):4713. doi: 10.3390/ijms23094713. PMID: 35563103; PMCID: PMC9102849.
7. Park HK, Ahima RS. Physiology of leptin: energy homeostasis, neuroendocrine function and metabolism. *Metabolism*. 2015;64(1):24-34. doi: 10.1016/j.metabol.2014.08.004. PMID: 25199978; PMCID: PMC4267898.
8. Kamel HFM, Nassir AM, Al Refai AA. Assessment of expression levels of leptin and leptin receptor as potential biomarkers for risk of prostate cancer development and aggressiveness. *Cancer Med*. 2020;9(15):5687-96. doi: 10.1002/cam4.3082. PMID: 32573960; PMCID: PMC7402836.
9. Kim HR. Obesity-related colorectal cancer: The role of leptin. *Ann Coloproctol*. 2015;31(6):209-10. doi: 10.3393/ac.2015.31.6.209. PMID: 26812923; PMCID: PMC4724700.
10. Caruso A, Gelsomino L, Panza S, Accattatis FM, Naimo GD, Barone I, et al. Leptin: A heavyweight player in obesity-related cancers. *Biomolecules*. 2023;13(7):1084. doi: 10.3390/biom13071084. PMID: 37509120; PMCID: PMC10377641.
11. Qie S, Diehl JA. Cyclin D1, cancer progression, and opportunities in cancer treatment. *J Mol Med (Berl)*. 2016;94(12):1313-1326. doi: 10.1007/s00109-016-1475-3. PMID: 27695879; PMCID: PMC5145738.
12. Li Y, Wei J, Xu C, Zhao Z, You T. Prognostic significance of cyclin D1 expression in colorectal cancer: a meta-analysis of observational studies. *PLoS One*. 2014;9(4):e94508. doi: 10.1371/journal.pone.0094508. PMID: 24728073; PMCID: PMC3984178.
13. Ahlin C, Lundgren C, Embretsén-Varro E, Jirström K, Blomqvist C, Fjällskog ML. High expression of cyclin D1 is associated to high proliferation rate and increased risk of mortality in women with ER-positive but not in ER-negative breast cancers. *Breast Cancer Res Treat*. 2017;164(3):667-78. doi: 10.1007/s10549-017-4294-5. PMID: 28528450; PMCID: PMC5495873.
14. Li J, Guo BC, Sun LR, Wang JW, Fu XH, Zhang SZ, et al. TNM staging of colorectal cancer should be reconsidered by T stage weighting. *World J Gastroenterol*. 2014;20(17):5104-12. doi: 10.3748/wjg.v20.i17.5104. PMID: 24803826; PMCID: PMC4009548.
15. Hegazy R, Hegazy A. Hegazy' simplified method of tissue processing (consuming less time and chemicals). *Ann Int Med Dent Res*. 2015;1(2):57-61.
16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. 2001;25(4):402-8. doi: 10.1006/meth.2001.1262. PMID: 11846609.

17. Handa K, Yamakawa M, Takeda H, Kimura S, Takahashi T. Expression of cell cycle markers in colorectal carcinoma: superiority of cyclin A as an indicator of poor prognosis. *Int J Cancer*. 1999;84(3):225-33. doi: 10.1002/(sici)1097-0215(19990621)84:3<225::aid-ijc5>3.0.co;2-a. PMID: 10371338.
18. Al-Maghrabi J, Mufti S, Gomaa W, Buhmeida A, Al-Qahtani M, Al-Ahwal M. Immunoexpression of cyclin D1 in colorectal carcinomas is not correlated with survival outcome. *J Microsc Ultrastruct*. 2015;3(2):62-7. doi: 10.1016/j.jmau.2015.01.001. PMID: 30023183; PMCID: PMC6014192.
19. Albasri AM, Elkablawy MA, Ansari IA, Alhujaily AS. Prognostic Significance of Cyclin D1 Over-expression in Colorectal Cancer: An Experience from Madinah, Saudi Arabia. *Asian Pac J Cancer Prev*. 2019;20(8):2471-6. doi: 10.31557/APJCP.2019.20.8.2471. PMID: 31450922; PMCID: PMC6852817.
20. Abd-El Raouf SM, Ibrahim TR, Hegazy AA. Expression and significance of ezrin and E-cadherin in colorectal carcinoma. *Int J Adv Res*. 2014;2:936-44.
21. Bardou M, Rouland A, Martel M, Loffroy R, Barkun AN, Chapelle N. Review article: obesity and colorectal cancer. *Aliment Pharmacol Ther*. 2022;56(3):407-18. doi: 10.1111/apt.17045. PMID: 35707910.
22. Caruso A, Gelsomino L, Panza S, Accattatis FM, Naimo GD, Barone I, et al. Obesity-related cancers. *Biomolecules*. 2023;13(7):1084. doi: 10.3390/biom13071084. PMID: 37509120; PMCID: PMC10377641.
23. Li C, Quan J, Wei R, Zhao Z, Guan X, Liu Z, et al. Leptin overexpression as a poor prognostic factor for colorectal cancer. *Biomed Res Int*. 2020;2020:7532514. doi: 10.1155/2020/7532514. PMID: 32596369; PMCID: PMC7292990.
24. Wangefjord S, Manjer J, Gaber A, Nodin B, Eberhard J, Jirström K. Cyclin D1 expression in colorectal cancer is a favorable prognostic factor in men but not in women in a prospective, population-based cohort study. *Biol Sex Differ*. 2011;2:10. doi: 10.1186/2042-6410-2-10. PMID: 21888663; PMCID: PMC3179695.
25. Jun SY, Kim J, Yoon N, Maeng LS, Byun JH. Prognostic potential of Cyclin D1 expression in colorectal cancer. *J Clin Med*. 2023;12(2):572. doi: 10.3390/jcm12020572. PMID: 36675501; PMCID: PMC9867305.
26. Erkasap N, Ozyurt R, Ozkurt M, Erkasap S, Yasar F, Ihtiyar E, et al. Role of Notch, IL-1 and leptin expression in colorectal cancer. *Exp Ther Med*. 2021;21(6):600. doi: 10.3892/etm.2021.10032. PMID: 33884038; PMCID: PMC8056113.
27. Al-Maghrabi JA, Qureshi IA, Khabaz MN. Expression of leptin in colorectal adenocarcinoma showed significant different survival patterns associated with tumor size, lymphovascular invasion, distant metastasis, local recurrence, and relapse of disease in the western province of Saudi Arabia. *Medicine (Baltimore)*. 2018;97(34):e12052. doi: 10.1097/MD.0000000000012052. PMID: 30142858; PMCID: PMC6113030.
28. Perumal K, Mun KS, Yap NY, Razack AHA, Gobe GC, Ong TA, Kuppusamy S, Rajandram R. A Study on the immunohistochemical expressions of leptin and leptin receptor in clear cell renal cell

carcinoma. *Biomed Res Int*. 2020;2020:3682086. doi: 10.1155/2020/3682086. PMID: 32802842; PMCID: PMC7424391.
29. de Candia P, Prattichizzo F, Garavelli S, Alviggi C, La Cava A, Matarese G. The pleiotropic roles of leptin in metabolism, immunity, and cancer. *J Exp Med*. 2021;218(5):e20191593. doi: 10.1084/jem.20191593. PMID: 33857282; PMCID: PMC8056770.
30. Chen C, Chang YC, Liu CL, Liu TP, Chang KJ, Guo IC. Leptin induces proliferation and anti-apoptosis in human hepatocarcinoma cells by up-regulating cyclin D1 and down-regulating Bax via a Janus kinase 2-linked pathway. *Endocr Relat Cancer*. 2007;14(2):513-29. doi: 10.1677/ERC-06-0027. PMID: 17639064.
31. Lin TC, Hsiao M. Leptin and cancer: Updated functional roles in carcinogenesis, therapeutic niches, and developments. *Int J Mol Sci*. 2021;22(6):2870. doi: 10.3390/ijms22062870. PMID: 33799880; PMCID: PMC8002181.
32. Palaiologos P, Chrysikos D, Theocharis S, Kouraklis G. The Prognostic value of G1 cyclins, p21 and Rb protein in patients with colon cancer. *Anticancer Res*. 2019;39(11):6291-6297. doi: 10.21873/anticanres.13839. PMID: 31704859.
33. Jeong WK, Baek SK, Kim MK, Kwon SY, Kim HS. Prognostic significance of tissue leptin expression in colorectal cancer patients. *Ann Coloproctol*. 2015;31(6):222-7. doi: 10.3393/ac.2015.31.6.222. PMID: 26817017; PMCID: PMC4724703.
34. Rivadeneira DB, DePeaux K, Wang Y, Kulkarni A, Tabib T, Menk AV, et al. Oncolytic viruses engineered to enforce leptin expression reprogram tumor-infiltrating T cell metabolism and promote tumor clearance. *Immunity*. 2019;51(3):548-560.e4. doi: 10.1016/j.immuni.2019.07.003. PMID: 31471106; PMCID: PMC6903394.
35. Jiménez-Cortegana C, López-Saavedra A, Sánchez-Jiménez F, Pérez-Pérez A, Castiñeiras J, Virizuela-Echaburu JA, et al. Leptin, Both Bad and Good Actor in Cancer. *Biomolecules*. 2021;11(6):913. doi: 10.3390/biom11060913. PMID: 34202969; PMCID: PMC8235379.
36. Cohen-Mekelburg S, Schneider Y, Gold S, Scherl E, Steinlauf A. Advances in the diagnosis and management of colonic dysplasia in patients with inflammatory bowel disease. *Gastroenterol Hepatol (N Y)*. 2017;13(6):357-62. PMID: 28690452; PMCID: PMC5495039.
37. Itzkowitz SH, Yio X. Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation. *Am J Physiol Gastrointest Liver Physiol*. 2004;287(1):G7-17. doi: 10.1152/ajpgi.00079.2004. PMID: 15194558.

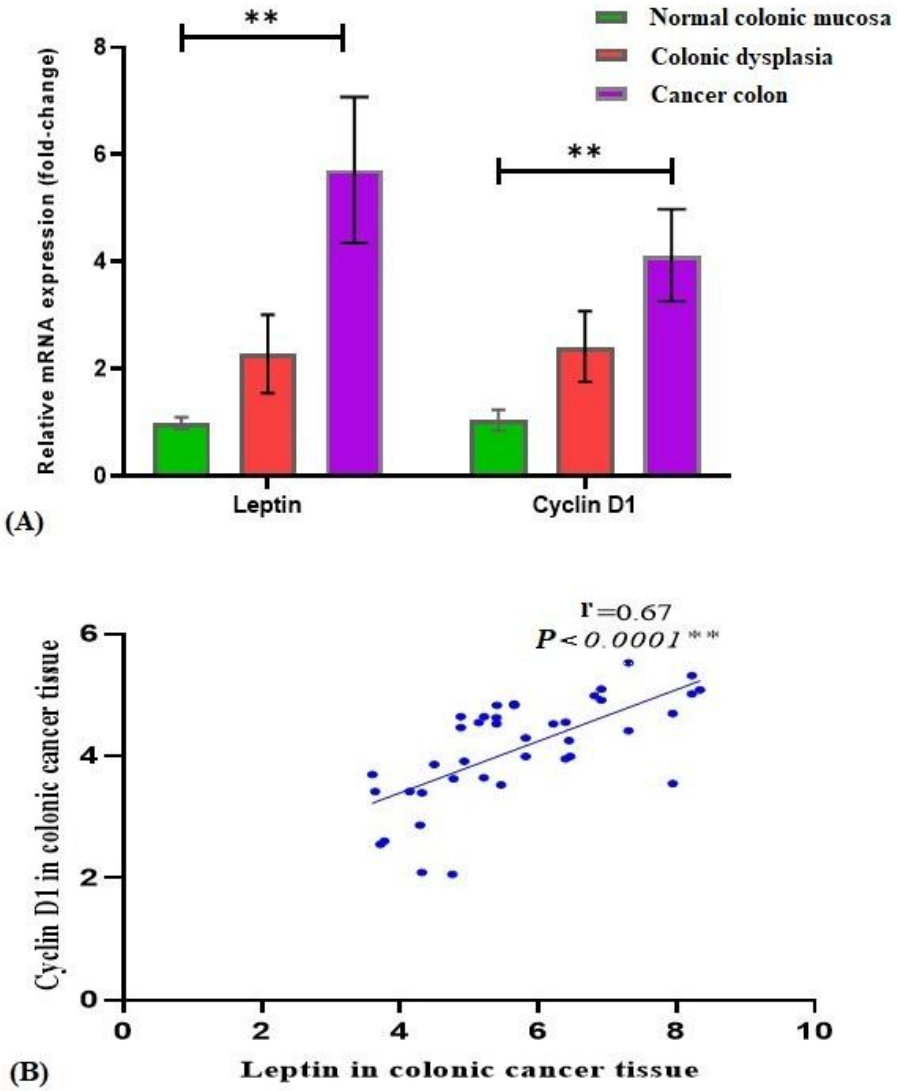

Figure 1. A) BAR chart shows leptin and cyclin D1 relative expression analysis in the studied groups expressed as mean ±SD, ** means $P < 0.001$ in comparison between normal colonic mucosa, colonic dysplasia, and colonic cancer specimens. B) Scatter plot shows positive association between leptin and cyclin D1 relative gene expression in the colonic cancer group with a Pearson correlation coefficient (r) of 0.67, $P < 0.0001$.

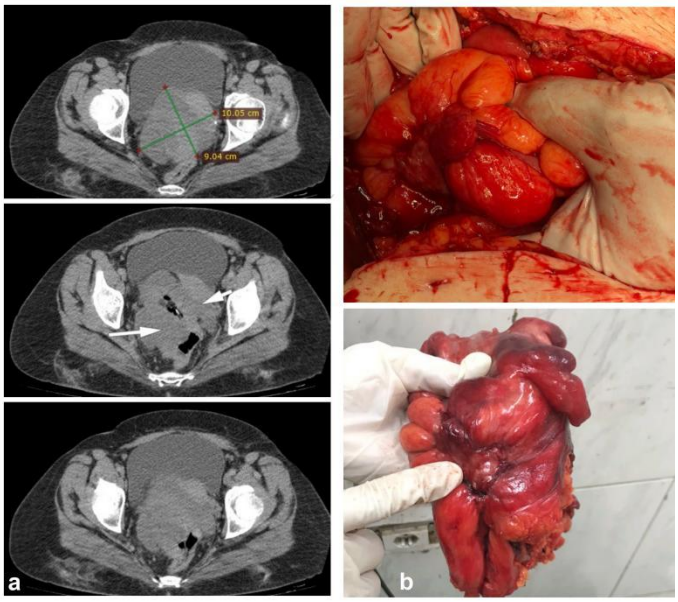

Figure 2. This figure shows the radiological and surgical images of sigmoid colon cancer. (a) Axial CT pelvis with contrast reveals hyperdense mass lesion in the sigmoid colon (white arrows) measures about 10x8 cm with narrowing of the lumen (T3 N0 M0). (b) Photography shows presence of large mass in sigmoid colon.
T3: Tumor 3; N0: Node 0; M0: Metastasis 0, CT: Computed tomography

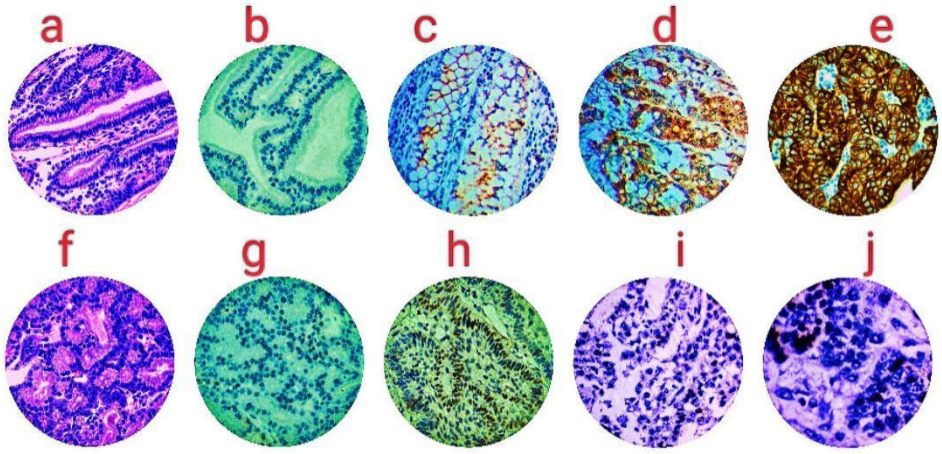

Figure 3. This figure shows the leptin and cyclin D1 immunohistochemical marker expression in different cases of normal and diseased colon cases. (1) Micrography shows normal colonic mucosal glands (H&E, 200×); (b) Micrography shows negative leptin IHC expression in adjacent normal colonic mucosa (Leptin IHC, 200×); (c) Micrography shows mild leptin IHC expression in a case of grade 1 well differentiated CRC (Leptin IHC, 200×); (d) Micrography shows moderate leptin IHC expression in a case of grade 2 moderately differentiated colonic carcinoma (Leptin IHC, 400×); (e) Micrography shows strong leptin IHC expression in a case of grade 3 poorly differentiated colonic carcinoma (Leptin IHC, 400×); (f) Micrography shows normal colonic mucosal glands (H&E, 200×); (g) Micrography shows negative cyclin D1 IHC expression in adjacent normal colonic mucosa (Cyclin D1 IHC, 200×); (h) Micrography shows moderate cyclin D1 IHC expression in a case of grade 2 moderately differentiated CRC (Cyclin D1 IHC, 400×); (i) Micrography shows moderate cyclin D1 IHC expression in a case of grade 3 poorly differentiated CRC (Cyclin D1 IHC, 400×); (j) Micrography shows strong cyclin D1 IHC expression in a case of grade 4 undifferentiated CRC (Cyclin D1 IHC, 400×).
H&E: Hematoxylin and Eosin; IHC: Immunohistochemical staining; CRC: Colorectal cancer

Table 1. Characteristics of the investigated patients (N = 60)

Variable		Total specimens N = 60	
		N = 60	**%**
Age in years (Mean ± SD)		49 ± 10	
Gender	Female	18	30.0%
	Male	42	70.0%
Obesity/ BMI ≥ 30	Absent	21	35.0%
	Present	39	65.0%
Groups (N = 60)	G1 (Normal)	8	13.3%
	G2 (CD)	12	20.0%
	G3 (CRC)	40	66.7%
		N = 40	**%**
Clinical presentations of CRC patients (N = 40)	Symptoms overlap*	5	12.5.0 %
	IDA	10	25.0 %
	Bleeding per rectum	5	12.5 %
	Changes in bowel habits	5	12.5 %
	Weight loss	10	25.0 %
	Intestinal obstruction/Perforation	2	5.0 %
	Abdominal mass	3	7.5 %

*Symptoms overlap (patients complaining of more than one of the above symptoms); N: Number; G: Group; BMI: Body mass index; CD: Colonic dysplasia; CRC: Colorectal cancer

Table 2. Comparison of leptin and cyclin D1 mRNA relative gene expression levels in the studied cases

Gene	Control (N = 8) Mean ± SD	CD (N = 12) Mean ± SD	CRC (N = 40) Mean ± SD	*P*-value	LSD
Leptin	0.98 ± 0.11	2.28 ± 0.73	5.71 ± 1.36	<0.0001**	0.04* (1) <0.0001** (2) <0.0001** (3)
Cyclin D1	1.04 ± 0.19	2.42 ± 0.66	4.12 ± 0.86	<0.0001**	0.0005*(1) <0.0001**(2) <0.0001**(3)

(1) G1 vs. G2, (2) G1 vs. G3, and (3) G2 vs. G3; **: Statistically highly significant difference ($P \leq 0.001$); *: Statistically significant difference ($P \leq 0.05$). *P* for ANOVA test; N: Number; CD: Colonic dysplasia; CRC: Colorectal cancer; SD: Standard deviation; LSD: Least significant difference post-hoc test

Table 3. Characteristics of the studied population as regard leptin and cyclin D1 mRNA expression

Variable	N.	Leptin	U	*P*	Cyclin D1	U	*P*
Age							
≤50	36	3.21 ± 1.92	94	<0.0001**	2.69 ± 0.30	118.5	<0.0001**
>50	24	6.18 ± 1.31			4.39 ± 0.66		
Gender							
Male	42	4.77 ± 2.02	275	0.09	3.63 ± 1.24	259	0.056
Female	18	3.53 ± 2.52			2.76±1.49		
Obesity (BMI ≥30)							
Absent	21	2.60 ± 2.02	126	<0.0001**	2.34 ± 1.42	157.5	0.0001**
Present	39	5.36 ± 1.69			3.92 ± 0.98		

The data expressed as mean ±SD, **: Statistically highly significant difference (*P* ≤0.0001), *P*-value for Mann-Whitney U test**;** SD: Standard deviation; BMI: Body mass index; N: Number

Table 4. Association of leptin and cyclin D1 mRNA expression and clinicopathological characteristics in colonic cancer patients

Characteristics	N.	Leptin	U	*P*	Cyclin D1	U	*P*
Histological Grade							
G1&G2	9	4.74 ±1.07	66.5	0.018*	3.83 ± 0.74	94	0.14
G3&G4	31	5.99 ± 1.31			4.21 ± 0.88		
Staging							
1&2	9	4.81 ± 1.13	70.5	0.026*	3.58 ± 0.92	76.5	0.043*
3&4	31	5.97 ± 1.32			4.28 ± 0.79		
Tumor size							
<5cm	15	5.02 ± 1.08	99.5	0.014*	3.82 ± 0.86	120.5	0.06
>5cm	25	6.13 ± 1.36			4.30 ± 0.82		
LN metastasis							
Negative	14	4.43 ± 0.59	19	<0.0001**	3.65 ± 0.83	84.5	0.005*
Positive	26	6.40 ± 1.13			4.38 ± 0.78		
Distant metastasis							
Negative	19	4.58 ± 0.61	4	<0.0001**	3.57 ± 0.85	55.5	0.0001**
Positive	21	6.74 ± 0.97			4.62 ± 0.50		

The data are expressed as mean ±SD, *: Statistically significant difference (*P:* <0.05), **: Statistically highly significant difference (*P:* ≤0.0001), *P:* -value for Mann-Whitney U test**;** N: Number; G: Group; LN: Lymph node

Table 5. Frequency of IHC leptin and cyclin D1 expression in the investigated cases (N = 60)

		Leptin		Cyclin D1	
		N	**%**	**N**	**%**
Negative		7	11.7%	8	13.3%
Positive	**Mild**	8	13.3%	9	15.0%
	Moderate	12	20.0%	20	33.3%
	Strong	33	55.0%	23	38.3%
Total	**Negative**	7	11.7%	8	13.3%
	Positive	53	88.3%	52	86.7%

N: Number; IHC: Immunohistochemical staining

Table 6. Characteristics of the studied groups as regard cyclin D1 and leptin expression

Variable		Cyclin D1				*P*-value	Leptin				*P*-value
		Negative N = 8		Positive N = 52			Negative N = 7		Positive N = 53		
		N	%	N	%		N	%	N	%	
Age (years)		43 ±8		49 ±10		0.128	41 ±6		49 ±10		0.046
Gender	Female	1	12.5%	17	32.7%	0.246	1	14.3%	17	32.1%	0.334
	Male	7	87.5%	35	67.3%		6	85.7%	36	67.9%	
Obesity (BMI ≥ 30)	Absent	5	62.5%	16	30.8%	0.08	5	71.4%	16	30.2%	0.032
	Present	3	37.5%	36	69.2%		2	28.6%	37	69.8%	
Group	G1 (Normal)	7	87.5%	1	1.9%	<0.001	6	85.7%	2	3.8%	<0.001
	G2 (CD)	1	12.5%	11	21.2%		1	14.3%	11	20.8%	
	CRC	0	0.0%	40	76.9%		0	0.0%	40	75.5%	

Quantitative data were expressed as mean ±SD and compared using independent T test, while qualitative data were expressed as numbers and percentages and compared using Chi-square X2 test; N: Number; G: Group; BMI: Body mass index; CD: Colonic dysplasia; CRC: Colorectal cancer

Table 7. Frequency of immunohistochemical markers co- expression of leptin and cyclin D1 in the studied cases

Markers		Cyclin D1				*P*-value
		Negative (N =8)		Positive (N =52)		
		N	%	N	%	
Leptin	Negative	7	87.5%	0	0.0%	<0.001
	Positive	1	12.5%	52	100.0%	

Variables expressed as numbers (N) and percentages (%) and compared using chi-square X2 test

Table 8. Association of cyclin D1 IHC expression and clinicopathological features of CRC (N = 40)

Variable		Cyclin D positive cases						Chi-square test	*P*-value	Leptin positive cases				Chi-square	*P* value
		Total		Low		High				Low		High			
		N	%	N	%	N	%			N	%	N	%		
Histological Grade (G)	**G1,2**	9	22.5	7	17.5	2	5	10.8524	0.000987*	8	20	1	2.5	10.2261	0.001385*
	G3,4	31	77.5	6	15	25	62.5			9	22.5	22	55		
Tumor size*	**1**	15	37.5	9	22.5	6	15	6.5934	0.010236*	10	25	5	12.5		
	2	25	62.5	5	12.5	20	50			4	10	21	52.5	10.5788	0.001144*
LN metastasis **	**0**	14	35	10	25	4	10	11.4004	0.003345*	8	20	6	15		
	1	26	65	5	12.5	21	52.5			3	7.5	23	57.5	9.4926	0.002063*
Distant Metastasis ***	**0**	19	47.5	13	32.5	6	15	8.0211	0.004624*	12	30	7	17.5		
	1	21	52.5	5	12.5	16	40			6	15	15	37.5	4.8211	0.028113*
Staging according to AJCC /2017	**1,2**	9	22.5	5	12.5	4	10			6	15	3	7.5		
	3,4	31	77.5	**5**	**12.5**	**26**	**65**	5.7826	0.016186*	**8**	**20**	**23**	**57.5**	**5.1188**	**0.023669***

*: Tumor size (1 ≤ 5cm, 2 >5 cm); **: LN metastasis (0 =absent, 1 =present); ***: Distant metastasis (0= absent, 1= present); G1,2: Groups 1 and 2; G3,4: Groups 3 and 4; LN: Lymph node; AJCC: American Joint Committee on Cancer