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RESEARCH TITLE

Differential Expression of CDX2 Gene in Colorectal Carcinoma: A Molecular Genetic Study

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Abstract

One of the common cancers in the world, which contributes a lot to cancer deaths, is colorectal carcinoma (CRC). Due to increased rates of incidence in the past few years, CRC has become one of the main health problems among Iraqis. The present study focused on determining the differences in the expression of CDX2 gene in colon cancer compared with benign and normal colon tissues. A total of fifty samples were selected, including twenty-two cases of colorectal cancer, twelve cases of benign tumours, and sixteen healthy individuals. Different hospitals were the source of the tissues used in the experiment, which had different ages between twenty and eighty-three. Extraction of RNA was made from the formalin-fixed paraffin-embedded tissues before being analysed in the lab. Reverse transcription followed by quantitative real-time PCR was performed to determine the expression of CDX2 gene with SYBR Green. According to the findings, expression levels of CDX2 were considerably reduced in CRC tissue specimens compared to benign and normal tissue specimens. It is plausible to consider that CDX2 can contribute to the growth and non-differentiation of tumors because the average expression level of CDX2 was lower in CRC samples. Statistical data confirmed that the difference between investigated groups was statistically significant ($P \leq 0.05$). In summary, the development and progression of CRC can be associated with the reduced expression of the CDX2 gene, which demonstrates the potential value of the examined gene as a marker for diagnostic and prognostic purposes. For the confirmation of obtained results, additional studies with increased numbers of participants should be performed.

Key Words: Colorectal carcinoma, CDX2, molecular biomarker. gene expression.

عنوان البحث

التعبير التفاضلي لجين CDX2 في سرطان القولون والمستقيم: دراسة وراثية جزيئية

المستخلص

يُعد سرطان القولون والمستقيم (CRC) أحد أكثر أنواع السرطان شيوعًا في العالم، كما يسهم بدرجة كبيرة في الوفيات الناجمة عن السرطان. ونظرًا إلى ارتفاع معدلات الإصابة به خلال السنوات القليلة الماضية، أصبح سرطان القولون والمستقيم إحدى المشكلات الصحية الرئيسة في العراق. هدفت الدراسة الحالية إلى تحديد الاختلافات في مستوى التعبير الجيني لجين **CDX2** في أنسجة سرطان القولون، مقارنةً بأنسجة الأورام الحميدة والأنسجة الطبيعية للقولون. اختيرت خمسون عينة، شملت اثنتين وعشرين حالة مصابة بسرطان القولون والمستقيم، واثنتي عشرة حالة مصابة بأورام حميدة، وستة عشر فردًا سليمًا. وجُمعت الأنسجة المستخدمة في الدراسة من مستشفيات مختلفة، وتراوحت أعمار المشاركين بين عشرين وثلاثة وثمانين عامًا. واستُخلص الحمض النووي الريبي (RNA) من الأنسجة المثبتة بالفورمالين والمطمورة في شمع البارافين، ثم خضعت للتحليل المختبري. وأُجري النسخ العكسي، متبوعًا بتفاعل البوليميراز المتسلسل الكمي في الزمن الحقيقي(RT–qPCR) ، لتحديد مستوى التعبير الجيني لجين **CDX2** باستخدام صبغة **SYBR Green.** أظهرت النتائج انخفاضًا ملحوظًا في مستويات التعبير عن جين **CDX2** في عينات أنسجة سرطان القولون والمستقيم مقارنةً بعينات الأنسجة الحميدة والطبيعية. ويشير انخفاض متوسط مستوى التعبير عن جين **CDX2** في عينات سرطان القولون والمستقيم إلى احتمال إسهام هذا الجين في نمو الأورام وفقدانها التمايز الخلوي. وأكدت البيانات الإحصائية وجود فروق ذات دلالة إحصائية بين المجموعات المدروسة عند مستوى دلالة .($P \leq 0.05$) وخلاصةً، قد يرتبط انخفاض التعبير عن جين **CDX2** بنشوء سرطان القولون والمستقيم وتطوره، مما يوضح القيمة المحتملة للجين المدروس بوصفه مؤشرًا لأغراض التشخيص والتنبؤ بمآل المرض. ولتأكيد النتائج المتحصل عليها، يُوصى بإجراء دراسات إضافية تشمل أعدادًا أكبر من المشاركين.

الكلمات المفتاحية: سرطان القولون والمستقيم؛ جين **CDX2**؛ المؤشر الحيوي الجزيئي؛ التعبير الجيني.

1. Introduction

Colorectal carcinoma (CRC), one of the leading factors for cancer-related sickness and death, is a major health concern around the world. CRC generally progresses in a multistep way, starting with adenomatous polyps and ending with invasive cancer. This cancer typically arises from the epithelial tissue of the colon and rectum [1,2]. Multiple behavioural, environmental, and genetic factors contribute to this transformation [3].

CRC cases increase with age, particularly in individuals above the age of 50, and are associated with many risk factors such as eating habits, obesity, and ongoing inflammation [4]. Even with advancements in screening and treatments, early diagnosis still remains a challenge, underscoring the importance of molecular biomarkers in diagnosing, prognosing, and treating patients [5].

The caudal type homeobox 2 (CDX2) gene encodes a transcription factor whose activity is important for the development of the intestine, epithelial cell differentiation, and maintaining intestinal function. The CDX2 transcription factor is considered to be important for establishing intestinal identity and is commonly found in the nuclei of intestinal epithelial cells [6]. In many cases of colorectal tumours, CDX2 is downregulated or absent, which is known to lead to undifferentiated, aggressive tumours with poor prognosis [7,8].

Analysing the expression pattern of CDX2 during the course of colorectal cancer could reveal valuable information considering the importance of CDX2 in preserving the intestinal epithelium. Therefore, the aim of this research is to assess the differential expression of CDX2 in tissue samples from colorectal cancer patients in comparison to normal colonic tissue samples and evaluate its role as a biomarker.

2. Material and methods

2.1 Samples collection

Between November 2025 and April 2026, 50 patients from Al Karama Hospital, Al Zahra Hospital, Baghdad Teaching Hospital, and the Digestive system Hospital had their colon and rectum samples collected.

2.2 Handling and Immediate Processing:

Tissue samples were immediately submerged in 10% buffered formalin after retrieval to achieve optimum fixing. The direction of resected samples was carefully marked, especially with bigger specimens. Sutures or dyes used as orientation markers helped to retain the histological environment and aided proper pathological interpretation later on.

2.3 Tissue Fixation

The preservation of cellular and tissue architecture is critical for correct histological evaluation. The fixation technique stabilizes tissue components, avoiding deterioration after sample.

2.4. Molecular studies of CDX2 gene in tumor colorectal

2.4.1.: -RNA Extraction and Deparaffinization

In this study, RNA extraction and deparaffinization were carried out in the following order:

Step 1: Solubilization of Paraffin A 1.5-ml Eppendorf tube has five sections, each 8 nm in size. To dissolve the paraffin, the slices were immersed in 1 ml of xylene for 5 minutes each time at room temperature. Following each treatment, the sections were subjected to a 10-minute high-speed centrifugation in a microcentrifuge. The pellet was preserved each time a centrifugation was done, and the supernatant was carefully removed without damaging the tissue fragments.

Step 2: Washing the Sample The tissue pieces were cleaned with 95 percent ethanol after a 10-minute wash with 1 cc of pure ethanol. After each wash, the samples were centrifuged for 10 minutes in a microcentrifuge, and the pellet was saved.

Step 3: Tissue Air-Drying The tissue slices were air-dried by leaving the tubes open in a 37°C thermoblock for around 30 minutes.

Step 4: Add Proteinase K Proteinase K was then applied to the dried tissues at a final concentration of 6 mg/mL. Proteinase K 20 mg/mL was added in the amount of 43 litres for every 100 liters of digesting solution.

Step 5: Incubation Overnight The treated tissues were incubated at 45°C overnight while being agitated to aid protein digestion.

2.4.2. RNA Extraction Process:

1. Homogenization of Samples The tissue sample was treated with 400 l of DR Buffer and

4 ul of ß-mercaptoethanol. The sample was then thoroughly homogenized using a tissue micro pestle.

2. Preparation of the Lysate and Grinding The pulverised tissue was placed in a 1.5 ml microcentrifuge tube, which was then filled with 400 l additional DR Buffer and 4 l more ß-mercaptoethanol. To shear the tissue, the lysate was injected ten times into a 20-G needle syringe.

3. Incubation of the Lysate and Centrifugation After incubating at room temperature for 5 minutes, the samples were centrifuged at 16,000 x g for 2 minutes. The supernatant was added to a GD Column in a 2 ml Collection Tube and centrifuged for 1 minute at 16,000 x g.

4. Collection Flow-Through The flow-through was saved for RNA purification after centrifugation, and the GD Column was placed in a new 2 ml collection tube and maintained at room temperature (15 to 25 ^{Co})

5. Elution of Buffer Heating Each sample's buffer (50 l) was warmed to 60°C.

Following the extraction step, the RNA was purified using the following methods:

6. Homogenization is the sixth step. After an overnight incubation, the digested tissues were homogenized using micro pestles to provide a uniform dispersion of the cellular components for the RNA extraction technique.

2.4.3: - RNA Purification Process:

1.Ethanol Addition: The flow-through was pipetted with 0.8 litres of absolute ethanol and well mixed. The sample was deposited in a 2 ml Collection Tube on the RB Column and centrifuged at 16,000 x g for 1 minute.

2.Buffer Addition and Centrifugation: The RB Column was held in place by a brand-new 2 ml Collection Tube. The sample was centrifuged for 30 seconds at 16,000 x g after 400 l of RW1 Buffer was added to the RB Column.

3.RPE Buffer Addition and Centrifugation: After filling the RB Column with 600 l of RPE Buffer, the mixture was centrifuged for 30 seconds at 16,000 x g after discarding the flow-through. This was repeated one more before drying the column matrix with a final centrifugation procedure at 16,000 x g for 3 minutes.

4.RNA Elution: -Finally, a clean 1.5 ml microcentrifuge tube was utilized to hold the RNase-free RB Column. 50 l of RNase-free water was put into the center of the column matrix and left to stand for 3 minutes. To elute the pure RNA, it was centrifuged at 16,000 x g for 1 minute.

2.4.4.: -Reverse Transcription

The Superscript First-Strand Synthesis System for RT-PCR was used for reverse transcription, and the Invitrogen procedure was followed. Here are the specific steps:

Step 1: Preparation of RNA/Primer Mixture The RNA/primer combination was produced as described in for each tube. (1):

Table 1: Components of RNA/primer mixture

Components	Amounts
RNA Total	5 g
Random hexanes (50 g/µl)	3 l
10 mM dNTP mix	1 l
DEPC H2O	to 10 l

Step 2: Reaction Mixture Addition: - The RNA/primer combination was then combined with the reaction master mixture, quickly mixed, and allowed to sit at room temperature for two minutes.

Step 3: Addition of Superscript II RT: - Each tube received one µl (50 units) of SuperScript II RT, which was added, mixed, and incubated at 25°C for 10 minutes.

Step 4: Incubation and Heating: -The tubes were heated at 70°C for 15 minutes after being incubated at 42°C for 50 minutes, and then they were cooled on ice.

Step 5: RNase H Addition: -The mixes received 1 µl of RNase H and 20 min of incubation at 37 °C.

Step 6: cDNA Storage: - When real-time PCR was required, the generated cDNA was kept at -20°C.

cDNA Synthesis

By adjusting the forward and reverse primer concentrations for each gene, cDNA synthesis was started. The ratio of forward and reverse primers in the mixture was fixed at 100 pM/ µl. The Stratagene MX3000p PCR software was utilised in accordance with the procedures described in Table 2.

Table (2) :PCR Program

Steps	Times	Temps	Cycles
1	2 min	50°C	1 cycle
2	10 min	95°C	1 cycle
3	15 s	95°C	40 cycles
	30 s	60°C	\
	30 s	72°C	\
4	10 min	72°C	cycle

2.4.5. Molecular detection of CDX2 gene: -

2.4.5.1. Dilution of primers: -

• Primer solutions (100 pmol/1 µl) were prepared by adding free NUECLASE water in exact amounts as required by the manufacturer.

● In order to achieve a final concentration of 10 pmol/1 µL, stock solutions of each primer were diluted with NUECLASE free water using a ratio of 1:9. Both forward and reverse primers were added in 1 µL volumes (10 pmol/1 µL). Gene Howard.

Table (3): Primers For Sequences of CDX2 gene: -

primers	Sequences
CDX2 F	5′CGGAACTGGAGAAGGAGTTC-3′,
CDX2 R	5′-TTCACTTGGGTCTCGTTGAG-3′.

2.4.5.2.: -PCR Working reaction: -

Components of **PCR** reaction and mixing amounts were shown in

table (4): **- PCR components**.

Component	Component of one sample
template cDNA	5 g l
Free of nuclease water	1
Primer in reverse	1 M
Primer for the future	1 M
Mastermix of Sibergreen	12 l
Volume total	20

The PCR Programs of CDX2gene: -

A. PCR programs of CDX2 gene: -

Table (5): -PCR program of CDX2gene: -

Steps	Time	Temperature	Cycles
Initial denaturation	10 min	95	1 cycle
Denaturation	30 sec	95	40 cycles
Annealing	40 sec	60	40 cycles
Extension	30 sec	72	40 cycles
Final extension	30 sec	25	1 cycle
Holding	Forever	4	-

2.5 Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 27 was used for data entry and analysis. Variables were presented using graphs and bar charts. Accordingly, mean differences of continuous numerical variables were assessed using independent samples t test and one-way ANOVA. The chi-square test was not used because more than 20% of the predicted cells had a value of less than 5; Instead, Fisher's exact test was used to evaluate the relationship between two categorical variables. Taking into account the significant P value, which is equal to or less than 0.05.

3. Results

The minimum age distribution of the 50 chosen patients was 20 years and the maximum age was 83 years, with a mean and standard deviation. figure 3.1

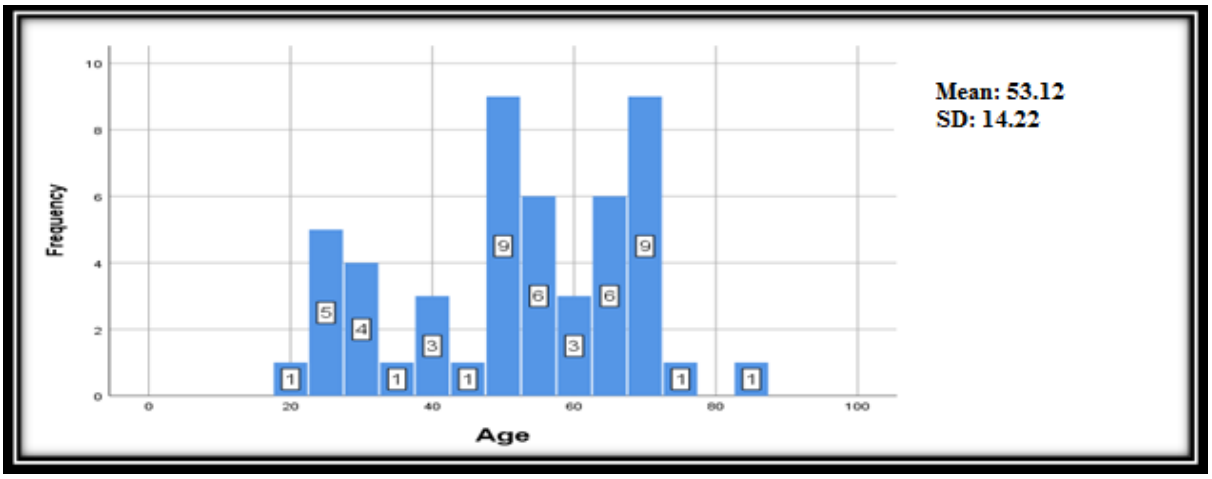


Figure 3.1 Distribution of age among the study.

Colorectal cancer patients included 22 individuals, accounting for approximately 43.3% of the sample. Figure 3.2 shows that there were 16 normal (control) patients and 12 patients (24.0%) with benign tumors.

Table 6. Association of sex of patients with group type among the three groups.

Sex	Group type			P-value
	Colorectal cancer	Benign	Normal	
Male	13 (43.3%)	9 (30.0%)	8 (27.7%)	0.357(Chi-square test)
Female	9 (45.0%)	3 (15.0%)	8 (40.0%)	

In table 6 age was significantly related to the patient's group (*P*-value=0.001). Eight out of 15 patients (53.3%) of patients aged 47-58 years were belonging to the CRC group. In addition to 50% of patients above the age of 60 years old were diagnosed with CRC. Normal peoples were mostly present in age groups of less than 43 years. There were 6 out of 10 (60%) aged between (20-33) years while 100% of those between 33-43 years are normal. Among those in the age group (59-71) years, there were 7 (43.8%) with benign diseases followed by 40% of patients aged 20-33 years who were also with a benign tumor. figure 3.2 shows that patients with CRC had the lowest mean 0.71 among the three groups when compared to benign and normal.

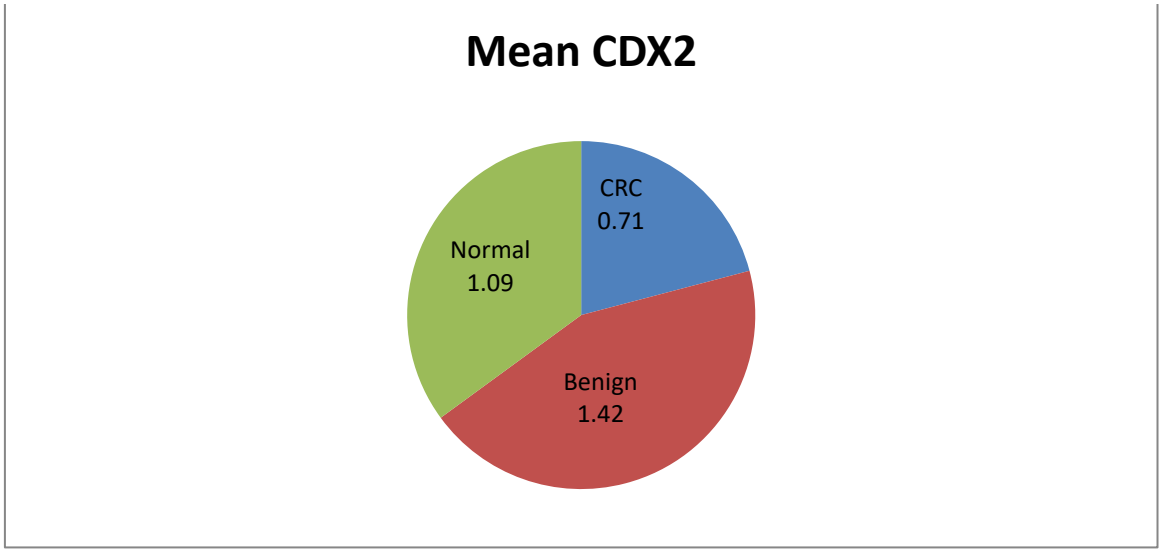


Figure 3.2 Means of CDX2 gene among the study's three groups.

In comparing CRC patients with normal people, table 6 significant differences were presented in means between the two mentioned groups for both studied genes (*P*-value < 0.001). For the CDX2 gene, the mean of normal patients was (1.09±0.24) which was higher when compared to CRC (0.71±0.20).

3.2. Histological changes in colorectal tumors

3.2.1 Normal mucosa to adenoma transformation.

Usually the first noticeable alteration, they are benign growths of glandular tissue. Depending on their structure, adenomas may have different sizes and shapes. These adenomas exhibit dysplasia, a disturbance in the typical maturation and arrangement of epithelial cells, when seen under a microscope. Dyplastic cells exhibit nuclear enlargement, hyperchromatism, irregular nuclear outlines, and an increase in the number of mitotic figures. Architectural anomalies that can be seen include glandular crowding, irregularity, and branching.[9.10].

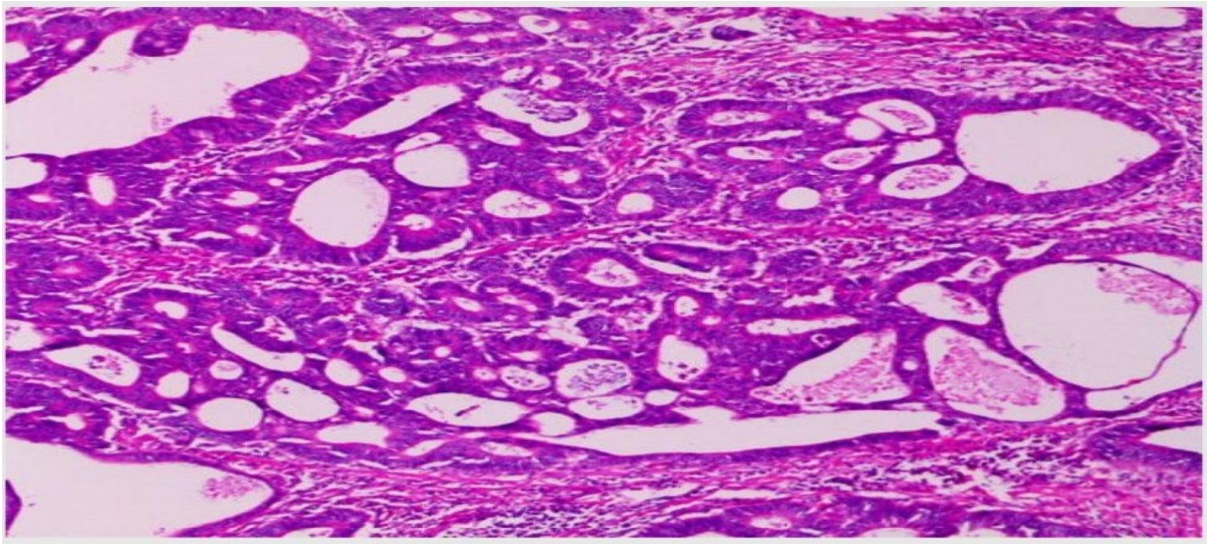
3.2.2. Adenoma to Carcinoma Progression

The degree of dysplasia rises with the size of the adenoma. This process causes an aggressive adenocarcinoma, the most prevalent form of colorectal cancer, to develop from a benign adenoma. The invasion of malignant glandular structures into the muscularis mucosa and beyond is what distinguishes adenocarcinomas[11]. Adenocarcinomas are usually composed

of malformed, aberrant glands that penetrate the stroma. The malignant glands are lined by pleomorphic cells with obvious nuclear abnormalities and numerous mitotic figures. A robust desmoplastic response, defined by an increase in stromal collagen, is typically observed around invasive tumor cells.[12].

3.2.3. Histological variants of colorectal adenocarcinoma.

While most colorectal tumors are classified as conventional adenocarcinomas, which include ring cell carcinomas, serrated adenocarcinomas, and mucinous adenocarcinomas [13].

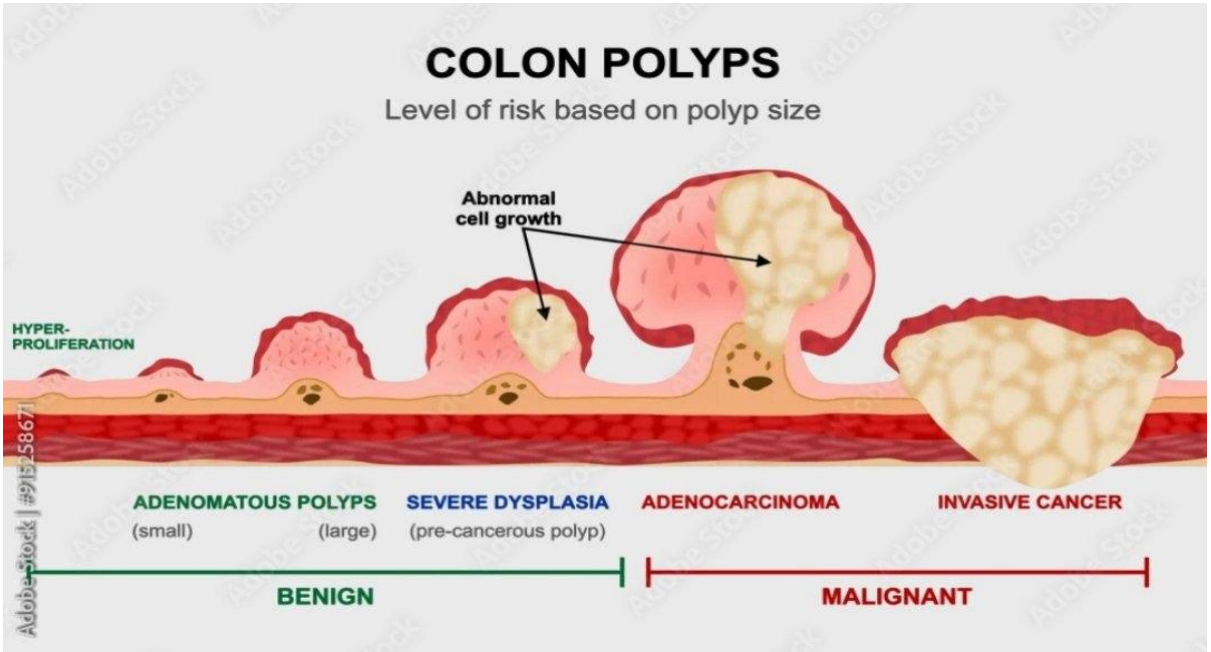


Moderately differentiated adenocarcinoma with muscularis propria invading muscularis propria in a 73 years old man in CA group T2 (H&F., 100X).

3.4.2. Histological staging of colorectal cancer

It is necessary to ascertain the disease's stage. The TNM staging approach, which considers the extent of tumor invasion, involvement of regional lymph nodes, and the existence of distant metastases, must be assessed when colorectal cancer is confirmed histologically. In addition to providing important information on the underlying molecular pathways of colorectal cancer, examining histological changes in colorectal tumors may help with diagnosis and therapy. As further research is undertaken, these findings may pave the way for novel therapeutic targets and methods in the fight against colorectal cancer [14,15].

Figure 3.4: adenomas Colon Polyps



4. Discussion

In the current investigation, we explored the role of CDX2 protein expression in normal colonic tissues, benign tumors, and CRC. In comparison to the two other groups, our results revealed a significant reduction in CDX2 expression in CRC. This result supports the hypothesis that CDX2 is vital for the maintenance of intestinal epithelial cell differentiation, and suppression of this protein promotes the proliferation of tumors.

CDX2 is an important transcription factor that regulates the expression of genes associated with differentiation and organization of epithelial tissue. Consequently, it explains the lower expression level of this protein in CRC tissues [16]. Loss of CDX2 function can cause the dedifferentiation of epithelial cells and result in a more malignant tumor phenotype, which makes it easier for metastasis.

The results obtained agree well with a number of other published investigations, which revealed low expression of CDX2 in colon carcinoma and its correlation with poor prognosis and advanced stages of tumors [16, 19]. This finding also emphasizes the potential usefulness of CDX2 as a diagnostic marker.

These results are consistent with numerous previously conducted investigations, according to which decreased expression of CDX2 was found in colon cancer, along with an advanced stage of tumors and poor prognosis [16, 19]. Another point emphasizing the importance of CDX2 as a diagnostic marker is associated with the variation of expression level in non-malignant versus malignant tissues.

5. Conclusion

As opposed to benign tumors and normal colon tissue samples, the current study found that the CDX2 gene expression level is much lower in colorectal cancer samples. According to the findings from this study, CDX2 is vital in ensuring proper intestinal epithelial cell differentiation, and any reduction in the expression of this gene can facilitate the progression of colorectal cancer.

Given the differences in the gene expression levels among the various study populations, it seems that CDX2 might be a very important biomarker for distinguishing malignant colonic lesions from non-malignant ones. Besides, the loss of cell differentiation and increased tumor aggressiveness may be related to the reduced expression of CDX2 gene.

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