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The Gene Expression of Cancer Stem Cells in Some Iraqi Patients with Colorectal Cancer

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Abstract: Colorectal carcinoma is the most prevalent gastrointestinal malignancy globally. The availability of statistics on cancer in Iraq is limited, however it is recognized as one of the leading causes of mortality after cardiovascular disorders. The initiation of carcinogenesis in colorectal cancer (CRC) occurs within a subset of cancer cells known as Cancer Stem Cells (CSCs), which possess the capacity to initiate and sustain tumor development and invasion. Lgr5, a Leucine-rich-repeated-containing G-protein–coupled receptor 5, is a controlled recipient of Wnt/b-catenin signaling. It was initially recognized as a potential indicator of colorectal cancer stem cells. This study comprised of fifty-five paraffin-embedded blocks, with 31 (56.36%) being males and 24 (43.63%) being females. These blocks were obtained from patients with colorectal adenocarcinoma. Among all the samples, 25 (45.45%) were moderately differentiated, 20 (36.36%) were poorly differentiated, and the remaining 18.19% had poorly differentiated colorectal carcinoma. The patients' ages spanned from 20 to 80 years, with a mean age of 50.6±7.44 SE.In Iraqi patients, there was a significant difference in the overexpression of the LGR5 protein among different grades (P value=0.015, r=0.919). The expression of the cancer stem cell biomarker Lgr5 did not show a significant association with gender (P value=0.143, r=0.425), age group (P=0.510, r=0.284), or lymph-vascular and perinural invasions (p=0.268,r=0.192). The results of the study indicate that there was a significant difference in the overexpression of Lgr5 amongst tumors of different sizes (P value=0.0001, r=0.885).

Key words: gene expression, cancer stem.

Introduction

The lack of data on cancer in Iraq is a significant issue, as it is one of the leading causes of death in our country, second only to cardiovascular diseases. This is mostly due to the aberrant proliferation of cells in the colon and rectum, which have the capacity to infiltrate and metastasize to other areas of the body. Colorectal cancer is the leading cause of mortality among internal organ malignancies and ranks as the seventh most prevalent cancer in Iraq, comprising 4.7% of all malignant tumors. It affects both genders equally. The occurrence of the event starts to increase from the age of 40 and reaches its highest point between the ages of 40 and 75, without any significant relationship with age. In Iraq, adenocarcinoma accounted for around 84% of all cases.

Cancer is not solely a genetic disorder. It is defined by unregulated cellular development and fast proliferation, which can lead to the invasion of neighboring tissues through the formation of metastases. Malignant tumors are comprised of many groups of cancer cells (Kozovska S, et al., 2014). The transformation of a healthy cell into a cancerous cell is a gradual process that entails the accumulation of numerous mutations in oncogenes and the deactivation of tumor suppressor genes. The colorectal carcinoma originates from the epithelial tissue of the colon, which is a location where several types of cancer can develop, such as sarcoma, melanoma, and lymphoma (Nicum et al., 2003). Colorectal Cancer (CRC) is the second most usually diagnosed cancer among females and the third most commonly diagnosed cancer among males. Despite advancements in detection and therapeutic procedures, colorectal cancer (CRC) continues to have a substantial impact on health-related mortality (Gustaw Lech, et al., 2016).The study of CRC continues to captivate researchers globally, owing to its high prevalence, progress in radiological imaging, and the capability to get biopsy samples for histological and genetic investigations (Mihalache AL., Rogoveanu I.,2011). The term "colorectal" refers to the large bowel, which is a muscular tube. The cecum is the initial segment of the large intestine, followed by the colon as the second segment, and the rectum as the final segment of the large intestine (Gartner and Hiatt, 2014). The large intestine, also known as the big bowel, is composed of a smooth mucosal membrane that lacks folds except in its distal (rectal) section and does not include villi (Mescher A. M, 2010).

The tubular intestinal glands penetrating throughout The mucosa that are lined by absorptive and goblet cells, with a scattered number of enteroendocrine cells, The colonocytes (absorptive cells) with short, irregular microvilli. Epithelium stem cells lie in the lower third of each gland (Mescher A. M,2010).

Simple columnar enterocytes (epithelial lamina) possess long microvilli line the surface of the mucosa, which is enclosed in a layer of mucus to facilitate the movement of the feces (Mescher A. M,2010). The lamina properia contains plasma cells , macrophages and other immune cells. On other hand, blood vessels, lymph nodes and specifically fat tissue located in the submucomsa. .Whereas only the taeniae coli essentially possess an outer longitudinal muscle system (Welsch, 2006). U-shaped segment of the large intestine, as follows: the ascending colon, the hepatic or right colic flexure, the transverse colon, the splenic or left colic flexure, the descending colon and the V-shaped sigmoid colon (Drenckhahn and Waschke, 2008 ; American Cancer Sociaty, 2015).

Methodology

Study population

This is cross section study group consisted of 55 samples of archival colorectal tissue that had been fixed with formalin and embedded in paraffin wax. Ages of patients had ranged from 20 to 80 years, with a mean of (50.66± 7.44 SE) years.

These cases were collected from laboratories of Histopathology in Alsadr Teaching Hospital in Najaf, Diwaniya and from four private laboratories in these governorates. Patients were submitted either for hemicoloectomy or endoscopic punch biopsy. Ethical approval for this study was obtained from ethical committee of Kufa University/College of Medicine.

The histopathological diagnosis was confirmed by reviewing freshly sectioned, H&E stained slides by two independent pathologists. Malignant tumors were reclassified according to type, grade and stage using TNM staging system (CAP,2016).

Quantitative Real-Time PCR (qPCR)

Analysis of the quantification of LGR5and AURKA genes expression will be done by the quantitative Real-Time PCR that normalized by housekeeping gene (GAPDH) in tumor and normal tissue samples this method was carried out according to method described by Liu et al., 2013 as following steps:

Preparation of paraffin embedded tissue samples colorectal carcinoma (Well, Moderate and poor differentiated) and normal colorectal tissue

Total RNA Extraction

RNA was extracted from the paraffin embedded tissue samples by using a commercial purification system (abm's ExCellenCT Lysis Kit) which summarized as follows:

1. Paraffin embedded samples prepared as 5µm from all categories by using microtome apparatus and put in eppendorf tube per reaction for lysis.
2. One µl of Protease was added into 5µm of paraffin embedded samples, then mix with 50 µl of room-temperature Lysis Solution, and mix content by pipetting 35 µl of the mixture up and down 5 times with avoid creating bubbles.
3. The lysis reactions Incubated for 10 minutes at 37°C.
4. One µl of Protease inhibitor mixed into the lysis reaction. To terminate lysis, add 5 µl of room-temperature Stop Solution, and mix content by pipetting 35 µl of the mixture up and down 5 times.
5. The mixture incubated for 2 minutes at room temperature. Longer incubation time can be used for a large number of samples, but do not leave the reactions at room temperature longer than 20 minutes after the addition of Stop Solution.
6. Lysates can be used as template in various downstream applications.

Estimation of RNA Concentration and Purity

The extracted total RNA was assessed and measurement by Nanodrop spectrophotometer (THERMO. USA), There were two quality controls were performed on extracted RNA. First one is to determine the quantity of RNA (ng/µL), the second is the purity of RNA by reading the absorbance in spectrophotometer at 260 nm and 280 nm in same Nanodrop machine as follow:

1- After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, RNA).

2- A dry Chem-wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 1µl of ddH2O onto the surface of the lower measurement pedestal.

3- The sampling arm was lowered and clicking OK to initialized the Nanodrop, then cleaning off the pedestals and 1µl of the appropriate blanking solution was added as black solution which is same elution buffer of RNA samples.

4- Then cleaning off the pedestals and 1µl RNA sample pipet for measurement.

5- Nanodrop spectrophotometer at 260 nm and 280 nm is determining the purity of RNA by reading the absorbance. Assessment of the purity of DNA and RNA is determined

Housekeeping gene primers selection

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene primer was used as a control to measure and know the effect of gene expression under study as a gene from Housekeeping gene.

Preparation of genes primers

This primer (IDT DNA , USA) were provided in lyophilized form, dissolved in sterile deionized distilled water to give a final concentration of 100 picomole/ µl as recommended by provider and stored in a deep freezer until use. Concerning monoplex PCR, forward and reverse primers were specific for these genes. All primers are designed according to Primer3 software program.

Table (1): The primers that encode of genes in our study

Primer name	Sequence (5'-----3')	Product size (bp)	**Reference**
LGR5	F: **CTCCCAGGTCTGGTGTGTTG** R: **GAGGTCTAGGTAGGAGGTGAA**	**149 bp**	**Current Study**
GAPDH	F:**CTGAGAACGGGAAGCTTGTC** R:**GAGAAGGCTGGGGCTCATTT GCA**	**154 bp**	**Current Study**

Amplification reaction of Housekeeping gene primers

LGR5, AUKA and GAPDH genes PCR mixture final volume 50µl was composed from 25µl of GoTaq®Green Master Mix (2x), 2µl template DNA, 2µl primers (for each forward and reverse), and 19µl nuclease free water in PCR tube. Eppendrof tubes then were briefly

Agarose gel electrophoresis

1- Agarose gels will always be made in 0.04M Tris-Acetate-EDTA, pH 8.3 (1X TAE) buffer.
2- Appropriate amount of agarose (a 1.5% gel would be 1.5g agarose in 100 mL of 1x TAE).
3- Make the mixture in a 250 mL flask, cover it with Saran Wrap, and microwave for 2-3 minute.
4- The gel allowed to cool a bit (5 minutes on the bench is usually sufficient).

3.0 µL of 10 mg/mL ethidium bromide was added to the flask, and then the gel was pour into the mold. Pour up to half the height of the teeth on the comb. Chase out any bubbles on the surface with a plastic Pasteur pipet or micro pipettor tip.

5- The gel allowed to cool until it turns slightly white. This usually takes at least 20 minutes at room temperature. the comb then Removed, and enough 1X TAE into the buffer chamber poured to barely cover the top surface of the gel.

6- Samples were loaded by mix 1.5 µl of sample with 1µl of green dye by pipetting 3-5 times until mix gently.

7- By using micropipette the mixture put in the holes and then power supply run initially at 70 volt for 5 min and then at 120 volt for 30 min.

Detection of Gene Expression by (qRT-PCR) system

Detection of gene expression by abm's One-Step Bright Green qRT-PCR Kit, is a complete qPCR system containing all necessary reagents for both reverse transcription and PCR amplification to occur in a single qPCR reaction tube.

Table (2): The components of reactive volume that used in qRT-PCR

Component	Reaction Volume	Concentration
	50 µl	
Total RNA	2.5 µl	50ng/ µl
Eva Green qPCR Master Mix	25 µl	1X
Reverse Transcriptase	0.5 µl	1X
Forward Primer	2 µl	25 picamole
Reverse Primer	2 µl	25 picamole
DEPC	18.5 µl	-

1- Reaction mixture Prepared in a qPCR tube on ice .

2- Gently mix and ensure all the components are at the bottom of the amplification tube.
Centrifuge briefly if needed.

3- The thermal cycler Programed that cDNA synthesis is followed immediately by qPCR amplification.

Table (3): The optimization of temperature that used in qRT-PCR instrument that used in our study.

Step	Temperature	Duration	Cycle(s)
cDNA Synthesis	57 C°	55 mins	1
Pre-Denaturation	95 C°	7 mins	1
Denaturation	95 C°	25 sec	45
Annealing	57 C° to 72 C°	25 sec	

DNase Preparation

The extracted RNA was treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme kit) and done according to method described by Promega company, USA.The mixture was incubated at 37C° for 30 minutes. Then, 1µl stop reaction was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme action.

RESULT

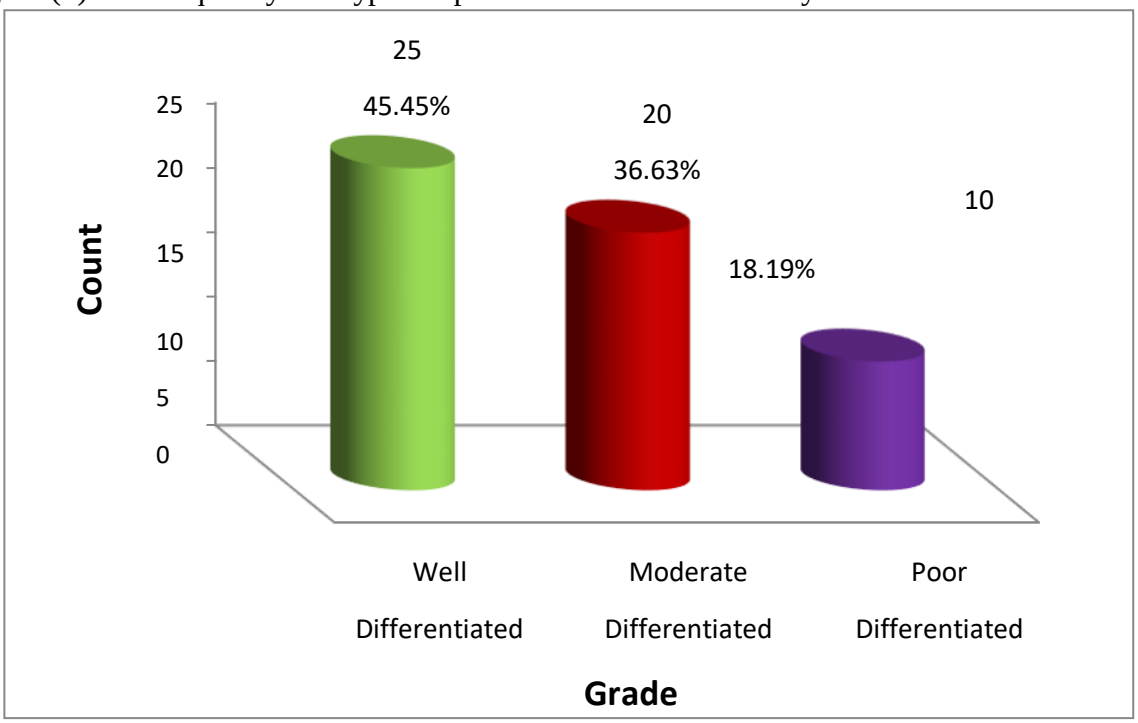
Clinic pathological characteristics in patients

Grading of the presented malignant cases were assessed according to American Society of Clinical Oncology (ASCO) in 2013.The distribution frequency of submitted cases in this study was as follow; 25(45.45%) had well differentiated colorectal carcinoma, 20(36.36%) had moderate differentiated colorectal carcinoma and (18.19%) had poorly differentiated colorectal carcinoma (Table 4-1) and (Figure 4-1).

Table (4): The frequency and types of presented cases in this study

No.	Types of colorectal Carcinoma	No. of cases	Percentage
1	*Well differentiated*	25	45.45%
2	*Moderate differentiated*	20	36.36%
3	*Poorly differentiated*	10	18.19%
	Total	**55**	**100.00%**

Figure (1):The frequency and types of presented cases in this study



The Distribution of Patients According to Gender

The clinicopathological assessment revealed that 31 (56.36%) of total colorectal carcinoma cases were males and 24 (43.63%) were females, with the male to female ratio was (1.29:1), showing no significant

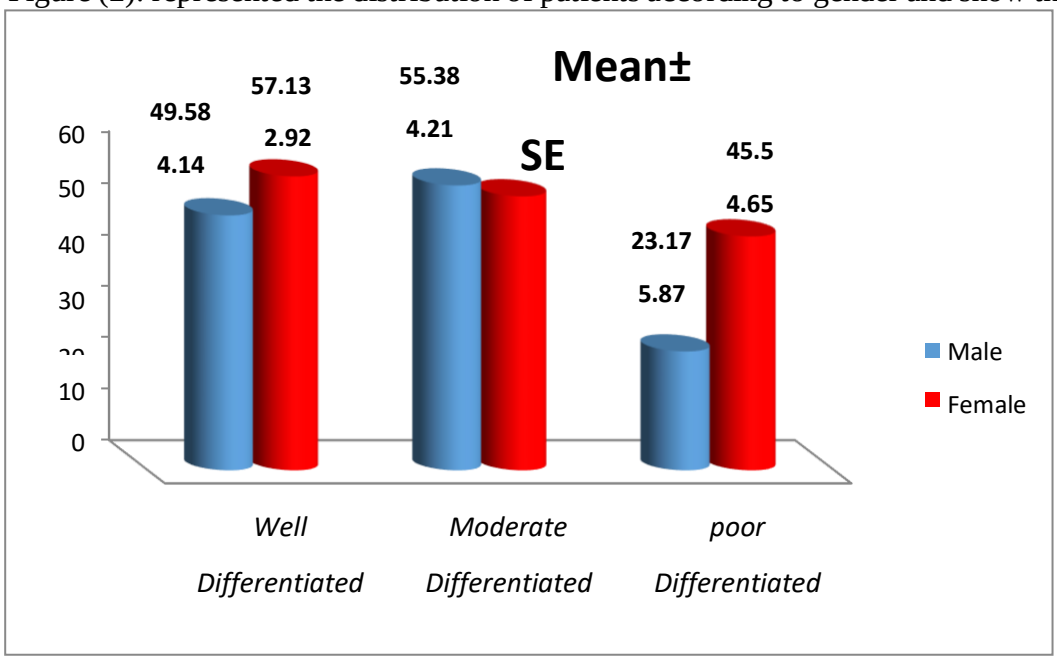
difference between male and female (P value = 0.443) (Table 4-2) and (Figure 4-2) .Colorectal carcinoma grades assessment revealed that non-significant difference between male and female at each group separated.

Table (5): The frequency and types of presented cases in this study

Carcinoma Group	Gender	No.	Mean	SE	Minimum	Maximum
well	Male	12	49.58	4.14	30	70
	Female	13	57.13	2.92	30	70
Moderate	Male	13	55.38	4.21	21	77
	Female	7	53.27	3.22	45	71
Poor	Male	6	23.17	5.87	22	59
	Female	4	45.5	4.65	33	55
***P*-value**	0.0443					

P value >0.05

Figure (2): represented the distribution of patients according to gender and show that Colorectal carcinoma



grades assessment revealed that non-significant difference between male and female at each group separated. (t-student test).

ns Well differentiated = p-value 0.1456.

ns Moderate differentiate= p-value 0.7409.

ns Poorly differentiated = P-value 0.3397

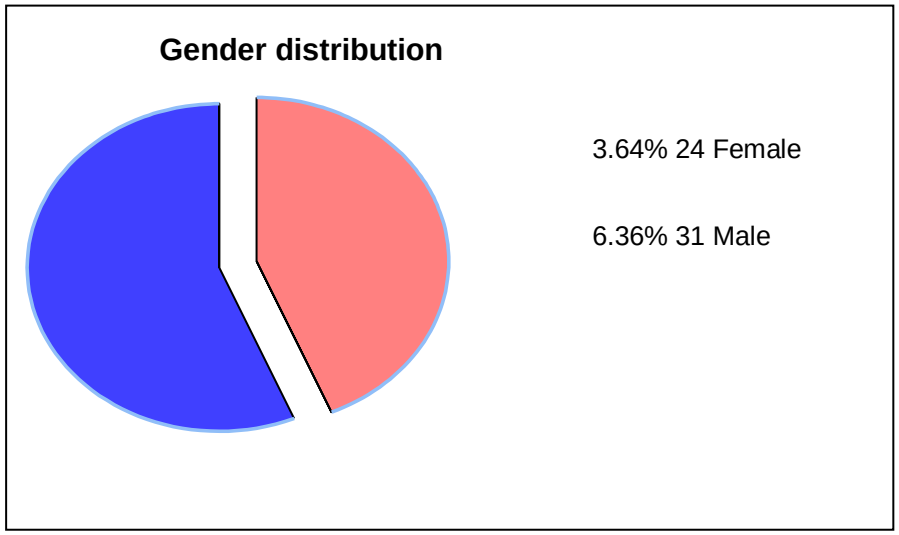


Figure 2 Gender distribution frequency of the presented colorectal tissue in this study

The Distribution of Patients According to age

The assessment of age distribution among colorectal carcinoma patients revealed the following findings: 6 patients (11.32%) were in the age group of 21-30 years, 4 patients (7.54%) were in the age group of 31-40 years, 13 patients (24.52%) were in the age group of 41-50 years, 18 patients (33.3%) were in the age group of 51-60 years, 8 patients (15.09%) were in the age group of 61-70 years, and 4 patients (7.54%) were in the age group of 71-80 years. The patients' ages ranged from 21 to 80 years, with a mean of 50.6±7.44 years (mean plus standard error). The references to Table 3-3 and Figure 3-4.

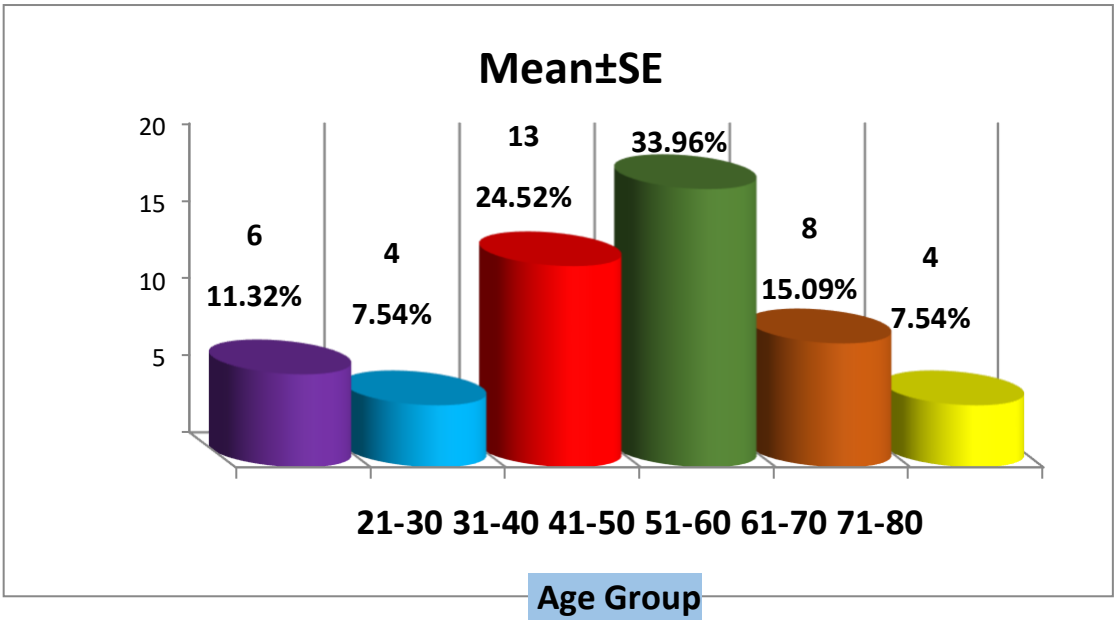


Figure (3): Age distribution of the presented colorectal carcinoma patients

Table (3): Age distribution of the presented colorectal carcinoma patients.

Age group	NO.	Median	%	Mean± SE	Minimum	Maximum
21-30	6	26.5	11.32	26±1.81	21	30
31-40	4	34.50	7.54	34.5± 1.04	32	37
41-50	13	48	24.52	47.46± 0.63	43	50
51-60	18	57.5	33.96	56.77±0.77	51	60
61-70	8	65	15.09	66±1.22	62	70
71-80	4	72.50	7.54	73.25±1.31	71	77
Total	**53**		**100**	**50.66 ± 7.44**		

The Distribution of Patients According to stages of CRC.

Assessment of the size (T) of the tumor of presented colorectal carcinoma according to the TNM staging system revealed that 11 (20 %) of T1, 25 (45.45%) of T2, while T3 formed 19 cases (34.55 %) (Table 3.4) and (Figure 3.5).

Table 4: The percentage of stage of the presented colorectal carcinoma

Types of Stage	No. of Cases	Percentage	*P value*
Stage T1	11	20.00%	
Stage T2	25	45.45%	
Stage T3	19	34.55%	
Total	55	100	0.6120

P value > 0.05

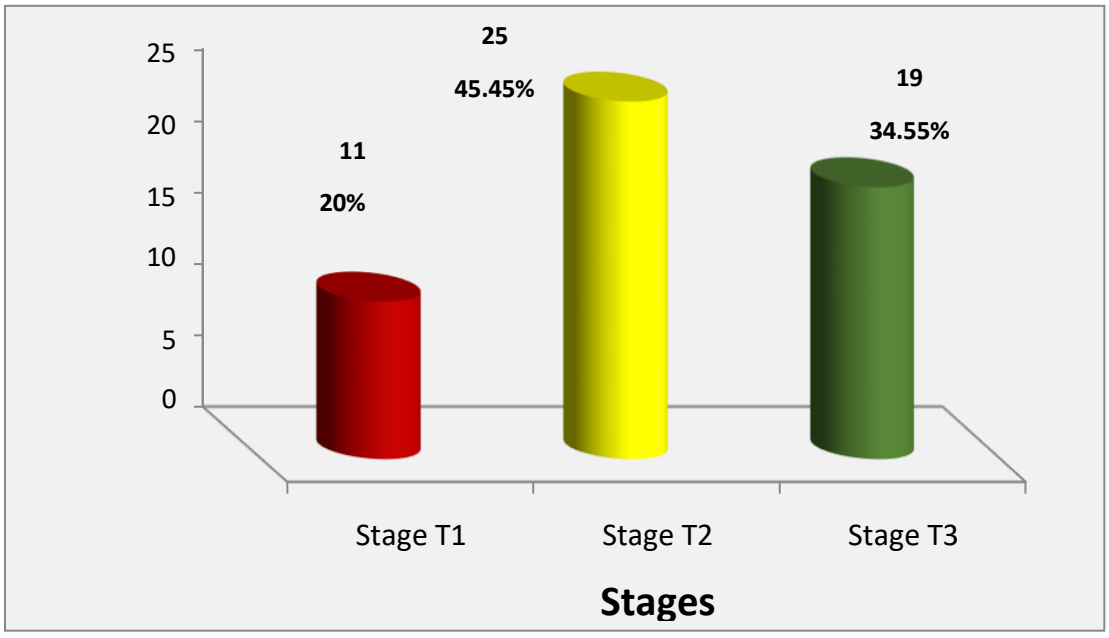


Figure (4 : The percentage of stage of the presented colorectal carcinoma 4-4 Molecular Study of Genes Expression

PCR&RT-PCR of LGR5 Gene Expression

The expression of LGR5 gene in colorectal carcinoma tumors was estimated in the 55 specimens of cDNA prepared from RNA of malignant colorectal adenocarcinoma tissues .The expression of LGR5 gene in colorectal carcinoma was achieved through the calibration against the expression of the same gene in normal tissues (calibrators). A normalized gene (GAPDH) was used as a control for the experimental variability in this quantification. Thus, the expression folds of LGR5 gene were calculated with respect to the internal control gene the housekeeping gene (GAPDH).

LGR5 gene amplification was reported in 37 (67.3%) cases out of 55 cases of colorectal adenocarcinoma and 18 cases were no change represented (32.7%). The expression (fold change) of LGR5 gene in colorectal carcinoma range between (6.393 ± 0.876) (Mean ±SE).

The Discussion

In the worldwide ,the colorectal carcinoma is the third most common cancer. The incidence start to increase at age 40 and until at age 75yr (Homady and Mariam.,2014). The men and women both face a lifetime risk of almost 6% for the development of invasive Colorectal carcinoma

.We can conclusion that colorectal carcinoma affected elderly people more commonly than younger people, and this result may be can explained depended on that carcinogenesis is a process of several steps that involve sequences of mutational processes on the levels of oncogenesand genes of tumor suppress, So the period of mutations genes may be need a long time by the effect of chemical carcinogens on colonic mucosa before the malignancy can be appear (American Cancer Society.,2017).

LGR5 is recently the most reliable recognized and utilized stem cell marker in the gastrointestinal tract and several other epithelial tissues and it is upregulated in a substantial fraction of solid tumors (Koo, B. K. & Clevers, H. ,2014). Other study showed that Lgr5 can be a valuable prognostic factor of Colorectal Cancer (CRC) Jiang etal.,(2016) but found that Lgr5 immunoexpression negatively correlated with tumor grade while our present study showed that Lgr5 immuno expression positively correlated with tumor grades and increased forward the progression of grade.

Alireza Mirzaei et al., (2015) reported in study that Lgr5 expression was no significant correlated with clinicopathologic finding of colorectal carcinoma patients such as sex, age ,tumor location as well as the lymphatic invasion was negatively correlated with Lgr5 expressionat p value (0.14)

,the same paper also showed that Lgr5 immuno expression was significantly in tumor grades, TNM stage , but showed no

significant differences in lymphatic invasion , this findings are solidified agree with our current study.

The lgr5 in present study also was positively expression associated with classic adenocarcinoma histology type and that clearly identical with Luka Stanisavljevic et al.,(2016) who and his colleagues also reported that is no significant differences between Lgr5 overexpression and age, sex of colorectal adenocarcinoma patients with p value (0.095) (0.442) respectively , but these studies showed that Lgr5 immunoexpression was no significant differences with tumor stage and Tumor differentiation and this no identical with our present study. The immunoexpression of cancer stem cell biomarker Lgr5 increased in CRC when comparison with non- tumors tissue and was significantly positively correlated with location, stage

,grade of the tumor ,lymph node and distant metastasis (Harb OA. et al.,2016) , but the same paper showed significant differences among gender and this disagreement with our present study. Harb OA. et al also found that tumor grades and stages positively correlated with grades and stages of colorectal carcinoma.

Song bing He et al.,(2014) also published paper talking about the Lgr5 overexpression and had no correlation with clinicapathological parameters such as age ,sex and BMI, also this results similar to our results obtained from this current study. Other studies concluded that Lgr5 was significantly higher in carcinoma than in normal mucosa and positively correlated with histological grade, depth of invasion, lymph node and pTNM stage (Wu et al.,2012.) and this showed the same findings of the present study in Iraqi people.

Given the tumorigenic role of Lgr5-positive stem cells in developed colon tumors, a pressing question is whether these cells with

Conclusions

The gene expression of cancer stem cell is associated with the aggressive and progression of the cancer .

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