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**RESEARCH ARTICLE**

# K-ras gene mutation and AgNOR score in colorectal carcinoma Iraqi patients

**Mariamm Mohammed Burhan [1], Dr. Merza Hamza Al-Murshedi [2]**

**1:** Babylon University/Faculty of medicine

**2:** Kufa University/ Faculty of science

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***Corresponding Author***

**Mariamm Mohammed Burhan**

## *Abstract*

**Background:**

In Iraq the incidence of colonic cancer is 4.5% . The conversion of a normal cell to a cancer cell is a stepwise process that typically involves the activation of oncogenes and inactivation of tumor suppressor genes (mutations). So the aim of the present study was to evaluate K-RAS mutation and the AgNOR score pattern and their relations to clinicopathoological aspects of colorectal carcinoma. The study included fifty tissue blocks embedded in paraffin wax (16 female and 34 males)which were obtained from patient group suffering from colorectal cancer (CRC) and thirty five Tissue blocks embedded in paraffin wax from normal colon (ulcerative colitis) which served as control group. AgNOR score was significantly higher in patients group than in control group ($P<0.001$). There was a highly significant positive correlation between grade of tumor and AgNOR score ($r=883$, $\mathbf{P}<0.001$), but there was no statistical association with age, gender, location of tumor and stage of disease. K-RAS mutation was not detected in all patients.

**Conclusions:**

AgNOR is significantly correlated with grade of colorectal carcinoma. K-RAS mutation was not detected in colorectal carcinoma



## INTRODUCTION

Colorectal cancer is a disease that is characterized by uncontrolled growth of colorectal epithelial cells. According to the theory of multistep carcinogenesis, colorectal cells accumulate a number of molecular changes and eventually become fully malignant cells. The earliest trigger genetic event is the inactivation of the adenomatous polyposis coli (*APC*) pathway. Mutations of tumor suppressor genes and oncogenes and likely several pathways accompany transitions in pathology of the lesions and drive tumor towards malignancy and metastasis (Bellacosa, 2003; Russo *et al*., 2009; Coppedè *et al.*, 2014). Colorectal cancer is not a single disease; rather, its encompass a heterogeneous complex of diseases with different sets of genetic and epigenetic alterations. ( Lindblom *et al.*, 2001; Vogelstein *et al.*, 2004; Leggett *et al.*, 2010).

Colorectal carcinoma is the third most common tumor in the western world (Haward *et al.*, 2005) and it is the second most common cause of death due to cancer. Colorectal cancer is the most common malignancy in men with 75 years and over The importance of colorectal carcinoma comes not only from that of it's big incidence but also from that it is a complete treatable disease if caught early (Boyle and leon, 2002).

More than one million individuals develop colorectal cancer (CRC) each year, and the disease specific mortality rate is in the developed world (Cunningham *et al.*, 2010; Labianca *et al.*, 2010). Colorectal cancer globally, is the third most commonly diagnosed cancer in both men and women. The highest incidence rates of CRC

are varies in Australia and New Zealand, Europe and North America, and the lowest rates are found in Africa and South-Central Asia (Jemal *et al.*, 2011).

There is a big geographical variation in the incidence of the disease over the world reaching 10 folds less in some Asian and African countries. In USA 146, 610 new cases were diagnosed as a colonic carcinoma and about 55000 of them died in the year 2006 (Nicum *et al.*, 2003; Bhatia *et al.*, 2000).

In Iraq the incidence of colonic cancer is 4.5% .Epidemiological risk factors may clarify this wide variation in the incidence of the disease (Iraq cancer registry, 2002).

Many immunohistochemical researches have focused on the colorectal carcinoma , studying the expression of various growth factors and gene expression ,including bcl-2(Saleh et al., 1999), CA125 (Pinto and Kotta, 1996),carbonic anhydrase (Kummola *et al.*, 2005).

**Aim of the Study:**

The aim of the study is designed to:

1- To determine if K-RAS mutation are frequent events in the colorectal cancer by: Detect genes expression by polymerase chain reaction (PCR) and Reflep-PCR method, through amplifying K-RAS gene sequence.

2- To correlate K-RAS mutation with other prognostic parameters such as age, sex, stage, and the grade of the tumor.

3. To study the significance of AgNOR score in colorectal carcinoma.

## Materials and methods

The present study was done in the molecular laboratory in the department of Biology, Faculty of Science, Kufa university, the molecular laboratory in the Faculty of Science, Babylon Universityand Al-Sadar Teaching Hospital in Al-Najaf province. The study was conducted from January 2014 through August 2015.

It included fifty tissue blocks embedded in paraffin wax (16 female and 34 males)which were obtained from patient group suffering from colorectal cancer (CRC) and thirty five Tissue blocks embedded in paraffin wax from normal colon (ulcerative colitis) which served as control group.

Each paraffin block was processed as following: Hand E stain for histopathological assessment, detection of Mutation in K-RAS by PCR and AgNOR score.

## Results

The mean age of patients group was 51.44±16.67 years and the median was 52 years. The age range in patients group was from 24 years through 85 years, whereas the mean age of control group was 45.86±15.73 and median age was 49 years. The age range in control group was from 10 to 65 years. Table 1 revealed that there was no statistical difference in mean age between control and patients groups (***P***>0.05), as shown in table 1.

**Table 1:** Showed parameters related to age regarding patients and control subjects

| Group | No. | Median | Mean | SD | Minimum | Maximum | P-value |
|---|---|---|---|---|---|---|---|
| Control | 35 | 49 | 45.86 | 15.73 | 10 | 65 | **0.124** |
| Colon carcinoma | 50 | 52 | 51.44 | 16.67 | 21 | 85 | |
| Total | 85 | 50 | 49.14 | 16.43 | 10 | 85 | |

The majority of patients with colonic carcinoma were more than 40 years of age, accounting for 80%, while 20 % of cases were below the age of 40 years.

The patients group included 34 males and 16 females, 68% and 32 % respectively, while control group included 21 males and 14 females, 60% and 40% respectively. Despite these minor differences in gender ratio between patients and control groups, there was no statistical difference (***P***>0.05), as shown in table 2.

**Table 2:** Showed the distribution of patients group and control group according to gender

| Gender | Control | | Colonic carcinoma | |
|---|---|---|---|---|
| | No. | % | No. | % |
| Male | 21 | 60.00 | 34 | 68.00 |
| Female | 14 | 40.00 | 16 | 32.00 |
| Total | 35 | 100.00 | 50 | 100.00 |

**P-value = 0.488**

**$X^2$ = 0.577**

**Df =1**

It was obvious that the a recto-sigmoid location is the most common site for colonic tumors in patients enrolled in the present study, as shown in table 3.

**Table 3:** Site of colonic carcinoma

| Site | No. | % |
|---|---|---|
| Recto-sigmoid | 30 | 60 |
| Right | 7 | 14 |
| Sigmoid | 7 | 14 |
| Left | 6 | 12 |
| Total | 50 | 100 |

It was clearly that the majority of patients exhibited well differentiated grade I tumors, as shown in table 4.

**Table 4**: Showing the classification of patients according to grade of tumor

| Grade | No. | % |
|---|---|---|
| I "Well differentiated " | 28 | 56 |
| II "Moderately differentiated " | 14 | 28 |
| III "Poorly differentiated " | 8 | 16 |
| Total | 50 | 100 |

The majority of patients were in stage II (66%), as shown in table 5

**Table 5:** Showing the distribution of patients according to stage of tumor

| Stage | No. | % |
|---|---|---|
| I | 11 | 22 |
| II | 33 | 66 |
| III | 6 | 12 |

| | | |
|---|---|---|
| Total | 50 | 100 |

The AgNOR score was significantly higher in patients group than in control group, 6.14±0.8 versus 1.31±0.12, ***P***<0.001, as shown in table (6). AgNOR positive stain was seen as variable sized almost oval black colored objects in tumor tissue figure (1) whereas in normal colonic mucosa the nuclear organizing regions were little in number and smaller in size figure (2).

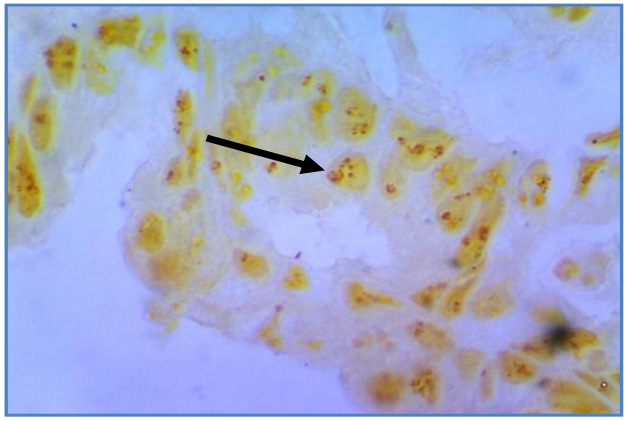

**Figure 1:** AgNOR stain in a colonic section from patient with colorectal carcinoma: nuclear organizing regions are seen as variable sized almost oval black colored objects (100X).

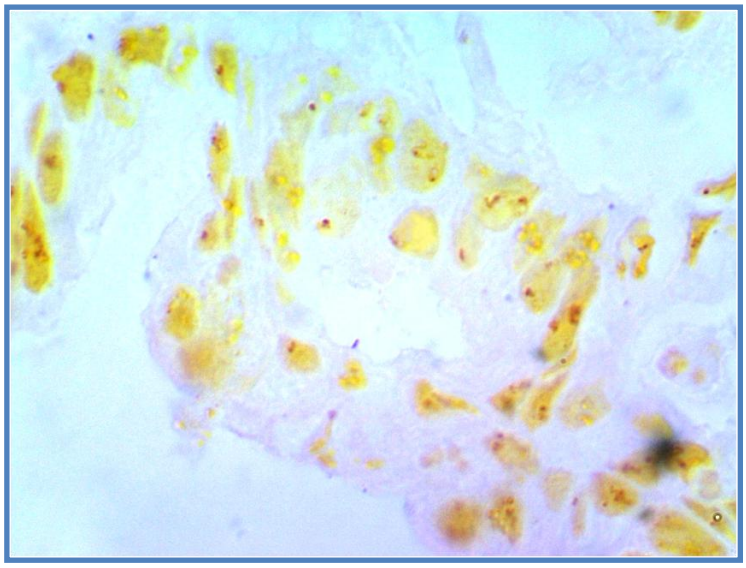

**Figure 2:** AgNOR stain in a normal colonic mucosa: nuclear organizing regions are seen as small sized almost oval black colored objects and their number is far less than that in case of colorectal carcinoma (100X).

**Table 6:** Showing the mean AgNOR score in patients group and control group

| Group | N | Mean | SD | P-value |
|---|---|---|---|---|
| Control | 35 | 1.31 | 0.12 | <0.001 |
| Colon carcinoma | 50 | 6.14* | 0.80 | |
| Total | 85 | 4.15 | 2.47 | |

No significant association was found between site of tumor and AgNOR score in patients with colonic carcinoma (***P***>0.05), as shown in table 7.

**Table 7:** Association between mean AgNOR and site of tumor

| Site | N | Mean | SD | *P*-value |
|---|---|---|---|---|
| Recto-sigmoid | 30 | 6.26 | 0.82 | 0.219 |
| Other sites | 20 | 5.97 | 0.76 | |
| Total | 50 | 6.14 | 0.80 | |

There was a highly significant positive correlation between grade of tumor and AgNOR score (r =883, ***P***<0.001), as shown in figure 3.

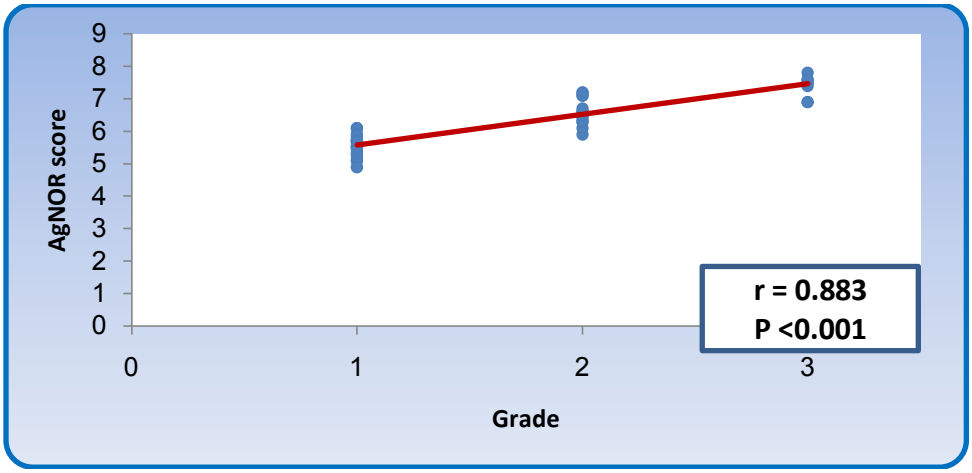


**Figure 3:** Showing the Spearman rank correlation between AgNOR score and grade

There was no significant correlation between AgNOR score and stage of disease (r = -0.001, ***P***>0.05), as shown in figure 4.

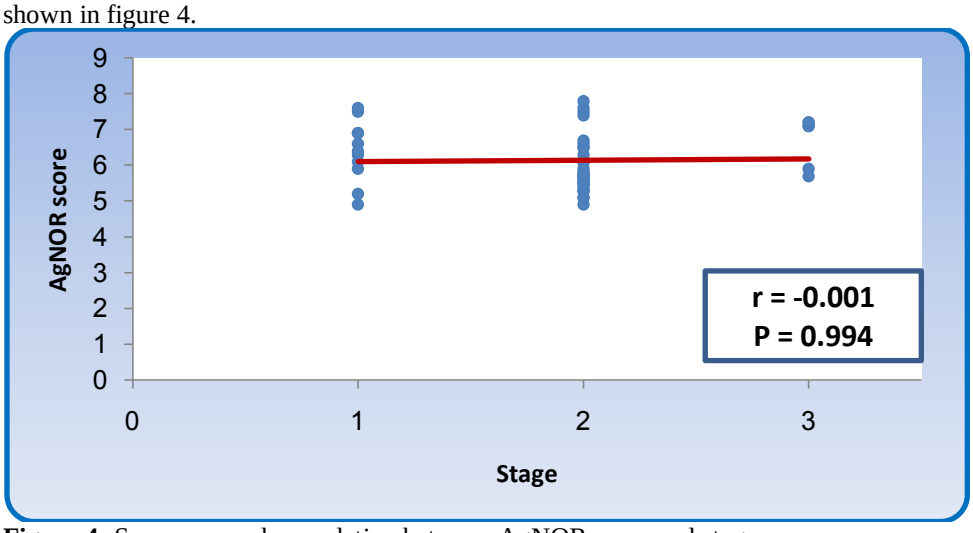


**Figure 4:** Spearman rank correlation between AgNOR score and stage

Colonic tissue obtained from all patients with colorectal carcinoma was proved to be negative for K-ras mutation by the use of PCR technique.

## Discussion:

In the present study the mean age of patients with colorectal carcinoma was 51.44±16.67 years and the median was 52 years while the age range was from 24 years through 85 years. On the other hand the majority of patients with colorectal carcinoma were more than 40 years of age, accounting for 80%, while 20 % of cases were below the age of 40 years.

Why does colorectal carcinoma affect elderly people more frequently than young age subjects?

This question can be discussed based upon the fact that carcinogenesis is a multistep process that involve a sequence of mutational events on the level of oncogenes and tumor suppressor genes. The etiology of cancer in general is attributed to two main general causes, the first one being hereditary germ line mutations in genes controlling cell cycle and growth and the second one is environmental factors. Germ line mutations are encountered in childhood tumors such as retinoblastoma in which a germ line mutation of the tumor suppressor gene (Rb) is claimed in the pathogenesis of such a tumor. This is off course not the case in colorectal carcinoma with exception of minority of cases that are attributable to familial syndromes. One can conclude that mutations in genes rendering colonic mucosa malignant are the result of environmental factors namely the interaction between products of food digestion and micro-organisms in the colonic mucosa. So a long period of time is needed for multiple genes to undergo mutation by the effect of chemical carcinogens on colonic mucosa before the appearance of frank malignancy, explaining the old age of the majority of patients.

According to Iraqi cancer registry (2009) the mean age of patients with colorectal carcinoma was 53.98+14.71 years and the median age was 55 years. These findings are comparable to the findings of the present study and the minor differences are clearly due to the difference in sample size which was 701 in Iraqi cancer registry while it was only 50 cases in the present study.

It was published in the Iraqi Cancer registry (2009) that about 82 % of colorectal carcinoma patients are above 40 years of age and that around 18% of patients are below the age of 40 years. This result is clearly similar to the finding of the present study. Talib *et. al*. (2009) stated that despite the clear relationship with aging, colorectal carcinoma is not strictly a disease of elderly and 6-8 % of cases occur in patients below 40 years of age (Talib *et. al*., 2009). **A**l-Humadi (2008) reviewed the records of 511 patients with colorectal carcinoma who were diagnosed in the period extended from 1965 through 1994 in Baghdad and stated that the median age was 50 years , a finding which strongly supported the finding of the present study. He also stated that 21.1% of cases occurred before the age of 40 years in accordance with finding of the present study. In another Iraqi study done in Kirkuk city, it was mentioned that the mean age of patients was 51.4 years (Summer and Osama, 2013), which is again similar to mean age obtained in the present study. Moreover another study done in Iraq stated that the mean age of patients with colorectal carcinoma was 54.5 years, a finding that solidified the result of the present study (Nidal *et.al*., 2014). Another Iraqi study done in Al-Kadhimiya teaching hospital on 94 colorectal cancer patients stated that about 24% of cases were under the age 40 years, a finding which is slightly higher than the finding of the present study which was 20%, but again it is in accordance with result of the present study (Hatam *et.al*., 2000). In another study done in Iraq, the mean age of patients with colorectal carcinoma was 52.4±16.3 years and an age range of 21-81 years (Suaad *et.al*., 2014); again these findings are similar to the findings of the present study. Additionally an Iraqi study was done in Duhok and stated that the mean age of colorectal cancer patients was 50.1 years and the age range was 20-80 years (Pity *et.al.*, 2013); these results again are in accordance to the results of the present study. Qasim *et. al*. (2012) stated, in a study done in Baghdad, that the mean age of patients with colorectal carcinoma was 56.03 and the age range 27-80 years; again these figures are approximately similar to the findings of the present study. Also Hashim *et. al.* (2010) stated , in a study done in Al-Najaf city that the mean age of patients with colorectal carcinoma was 58.1 years; this finding is higher than that of the present study but it is still in the sixth decade.

In a recent study done in Kuwait, on 116 patients with colorectal carcinoma, the mean age was 64.2 and the age range was 35-94 years (Ali *et.al*., 2014); these findings are substantially higher than that of the present study. The younger age of Iraqi patients with colorectal carcinoma in comparison to Kuwaiti patients may be explained by the presence of environmental carcinogenic agent in Iraq which facilitates the rate of developing colorectal carcinoma, which is not found in Kuwait.

In a study done on 4201 cases of colorectal carcinoma in the Kingdome of Saudi Arabia, the mean age of patients with colorectal carcinoma was 58 years and 79% of cases were above the age of 45 years (Mosli and Al-Ahwal, 2012); although the mean age is higher than that obtained in the present study, it is still in the sixth decade; from another point of view the finding that majority of cases were above 40 years of age is comparable to the finding of the present study. In a recent study done in Jordan on 241 colorectal cancer patients, the mean age was 56.7±13.6 years (Abu-Helalah *et.al*., 2013), which is slightly higher than that of the present study. The same reason

that has been mention earlier may apply here; younger Iraqi patients may suggest their exposure to a potential environmental carcinogen that is lacking in Jordan environment.

In a recent study done on 968 cases with colorectal carcinoma in Turkey, the mean age was 58.9±12.6 years which is higher than that of the present study, while the age range was 18-85 years which is comparable to the age range of the present study (Aykan *et.al*., 2015). In an Iranian study done on 546 patients with colorectal carcinoma, mean age was 55.2±11.5 years which is slightly higher than that of the present study, while 23% of those Iranian patients were under the age of 40 years, a figure that is slightly higher than that obtained in the present study (Mahmodlou *et. al*., 2012). Again this younger age of Iraqi patients with carcinoma in comparison to Turkish and Iranian patients may suggest the presence of an environmental carcinogenic agent in Iraq that made the progression of colorectal carcinoma faster and to appear in younger age persons.

In the United States the median age at time of diagnosis of colorectal carcinoma is 69 (American Cancer Society 2011) which is much higher than median age obtained in the present study and even higher than that of nearby countries as shown in the discussion above.

The discussion section concerning age, which was stated above, came up with an important question that need to be answered; why does Iraqi people develop colorectal cancer younger than nearby countries and much younger than United States population?

This question can be viewed from two points of view; the first one is racial variation in age incidence and the second one is the presence of environmental factor in Iraqi environment that is not present in other countries, exposure to radiation for instance. Racial variation may be an acceptable cause in case of United States, but off course it fails to explain the mean age difference between Iraqi patients and patients from nearby countries who have common Asian ancestors. The concept of therapeutically radiation induced colorectal carcinoma has been discussed by Rapiti *et.al*.(2008) who concluded a significant increase of colorectal carcinoma rate in patients treated by radiotherapy for prostatic carcinoma. Yang *et.al*. (2008) also reported five patients (one male and four females) who received radiotherapy for cervical and bladder carcinoma.

Military radiation as a cause of colorectal carcinoma has been investigated by Kaiser *et.al.* (2014) who proved association between radiation exposure and predisposition to colorectal carcinoma. The risk of colon cancer was found to increase significantly with increasing radiation dose in both Hiroshima and Nagasaki, and also in both males and females (Shigematsu and Kagan, 1986). Busby *et.al*. (2010) observed an increased incidence of birth defects and cancers that were attributed to depleted uranium usage in Iraq by US army. Radiation exposure during the first and second gulf war may explain in part the relatively younger age of colorectal carcinoma patients in Iraq in comparison with nearby countries.

Male to female ratio was 2.12 : 1 in the present study. Why is colorectal carcinoma more common in males than females? One of the hypotheses to answer this question is that females have estrogenic hormonal level much more than that owned by males. If this is true how does estrogen protects again colorectal cancer?. Hartman and Gustafsson (2010) concluded that estrogens are important in protecting against Colorectal carcinoma initiation and progression, and that the protective effect most likely is mediated by estrogen receptor β (ERβ).

The report of Iraqi cancer registry (2009) stated that the male to female ratio of patients with colorectal carcinoma was 1.23 : 1 which as the present study gave a higher incidence in male patients than female patients. Talib *et. al.* (2009) reported a male to female ratio of 1.6 : 1 which is slightly higher than that of the present study but again it emphasizes that male are affected more frequently than females. Al-Humadi (2008) reported a male to female ratio of 1.4 : 1 which is again, in accordance with the present study, clarified a higher frequency of colorectal carcinoma among male patients. Summer and Osama (2013) stated that male to female ratio among patients with colorectal carcinoma was 1.2:. Although this ratio is lower than obtained in the present study, it again showed a higher frequency of colorectal carcinoma in males than females. Nidal *et. al*. (2014) reported a 1.24:1 male to female ratio, which is lower than that of the present study; nevertheless it solidified the fact that colorectal cancer is more frequent in males than females. Male to female ratio was 1.5: 1 in the study conducted by Hatam *et.al.* (2000), which is exactly the same as that obtained in the present study. Suaad *et.al*. (2014) reported a male to female ratio of 1.27:1, which again emphasized the fact that colorectal cancer is more frequent in male patients. Qasim *et.al.* (2012) reported a male to female ratio of 1.35:1, which is slightly lower than that obtained in the present study, but still in accordance with most literatures that emphasized the higher frequency of colorectal carcinoma in males than in females. Hashim *et.al.* (2010) reported a male to female ratio of 1.08:1. In a study done in Kuwait the male to female ratio was approximately 1:1, which is different from that obtained in the present study (Ali *et.al.*, 2014). In a study done in Saudi Arabia the male to female ratio was 1.18:1; although being less than that of the present study, it still clearly document the higher frequency among male patients (Mosli and Al-Ahwal *et.al*., 2012). In the study that was conducted in Jordan the male to female ratio was 1.1:1, which is less than that of the present study, nevertheless it showed a higher incidence among male patients (Abu-Helalah *et.al*., 2013). In a study done in turkey the male to

female ratio was 1.56: 1, which is approximately similar to the ratio obtained in the present study (Aykan *et.al.*, 2015). In Iran the male to female ratio of colorectal cancer patients was 1.28:1 (Mahmodlou *et.al.*, 2012). In the United States Male rates were higher than female rates at all subsites for all racial/ethnic groups (Murphy *et.al.*, 2011).

The present study showed that the majority of colorectal carcinoma (56%) had a well differentiated grade I histological pattern. According to Ackerman surgical pathology textbook (2011), the usual malignant tumor of the large bowel is a well-to-moderately differentiated adenocarcinoma secreting variable amounts of mucin (Rosai and Ackerman, 2011).

Mahmodlou *et.al.* in (2012) reported that majority of colorectal carcinoma cases (64%) were well differentiated grade I lesions. This result is similar to the result of the present study.

Well differentiated morphology was the major histological grade reported by some authors: Talib *et.al*., 2009; Summer and Osama 2013 and Qasim *et.al.*, 2012.

Well differentiated grade I histological pattern is characterized by glandular pattern of growth with multilayering, loss of polarity, back to back arrangement and atypical nuclear features in the form of hyperchromatism, high nuclear to cytoplasmic (N/C) ratio and visible nucleoli.

The present study showed that majority of patients enrolled in the present study (66%) had stage II disease. This can be attributed to the fact that early stage ( carcinoma in situ and stage I disease) tumors are often not diagnosed due to lack of proper screening programs like colonoscopy and imaging techniques which can be applied on high risk groups. As it is difficult to cure patients with advanced stage tumors, it is mandatory to apply screening programs for early detection of these common tumors in Iraq.

The finding of the present study that that majority of patients had stage II disease is in agreement with many authors: (Summer and Osama , 2013), (Hatam *et.al.*, 2000) , (Qasim *et.al*., 2012), (Hashim *et.al.*, 2010), (Ali *et.al.*, 2014), (Abu-Helalah *et.al*., 2014) and (Mahmodlou *et. al*., 2012).

The Kirsten Ras (*KRAS*) proto-oncogene encodes for a guanosine triphosphate (GTP)/guanosine diphosphate binding protein downstream of the epidermal growth factor receptor (EGFR) in the RAS/RAF/MAPK pathway. Mutations in *KRAS* are evident in 30–40% of colorectal tumours (Andreyev *et al*, 1998; Samowitz *et al*, 2000; Gnanasampanthan *et al*, 2001; Wang *et al*, 2003; Lee *et al*, 2008; De Roock *et al*, 2010; Nash *et al*, 2010; Roth *et al*, 2010; Hutchins *et al*, 2011; Imamura *et al*, 2012; Inoue *et al*, 2012). Based on evidence that the benefits of adjuvant treatment with anti-EGFR chemotherapy for distant-stage metastatic colorectal cancer (CRC) are limited to patients with *KRAS*-wild-type disease (Lin *et al*, 2011; Bokemeyer *et al*, 2012), testing for *KRAS* mutations is increasingly common in clinical practice in order to better direct treatment of CRC (Allegra *et al*, 2009).

In the present study none of the tissue samples taken from the paraffin block of patients with colorectal carcinoma was positive for K-ras mutation. This may be due poor storage of paraffin blocks or due to the effect of formalin fixation. Sample size also may be blamed and increasing sample size in future studies with the use of fresh tissue may help in estimating the real rate of K-ras mutations in Iraqi patients with colorectal carcinoma.

The present study showed that AgNOR score was significantly higher in patient group in comparison with the control group and also it was significantly correlated with the grade of tumor, being higher with less differentiated tumors. These findings can be attributed to the fact that malignant tumor cells are often characterized by aberrant chromatin pattern like plyploidy and aneuploidy, and this abnormal chromatin pattern will be reflected in the form of high score nuclear organizing regions.

Wang and Zhang (1995) reported that Counting on the mean number of AgNOR showed a predominant difference between the tumor tissue (9.796 +/- 3.670) and normal mucosa (2.937 +/- 0.812), ($P < 0.001$). With regard to the tumor differentiation, the number of AgNOR was paralleled with the degree of malignancy of the tumor, 8.178 +/- 2.864 for well differentiated tumors, 10.086 +/- 3.940 for moderately differentiated tumors and 11.577 +/- 2.824 for poorly differentiated ones. These findings supported the result of the present study. Eminovic-Behrem *et al.* (2000) stated that there was a significant correlation between AgNOR count and degree of tumor differentiation in patients with colorectal carcinoma. Nishi *et.al*. (1994) reported than the mean count of AgNOR was significantly higher in tumor tissue in comparison with normal mucosa; this observation was made upon a study conducted on 47 colorectal carcinoma patients. This result confirmed the result of the present study. Zhou and Yuan (1995) also reported a significant association between AgNOR count and grade of colorectal tumors.

These findings explored a prognostic value for AgNOR as its correlation with grade of tumor may be reflected on survival.

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