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Molecular study of colorectal carcinoma and *EGFR* gene mutations

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ABSTRACT

The development of various human tumors can be related to the activation of the Epidermal growth factor receptor (*EGFR*) and its subsequent signaling pathways. There are so much alertness and awareness that has been given to the *EGFR* pathway recently because *EGFR* and some downstream components together render as targets for anticancer therapy. The *EGFR* pathway and its impact on colorectal carcinogenesis and assessments are the assertiveness in this paper. In this study, we took 1034 patients with colorectal carcinoma that were recorded as a medical survey we used a standard questionnaire for those patients and we used real time PCR for 30 patients from 134 cases that have colorectal carcinoma to detect if there is any mutation in the *EGFR* gene. We chose 4 exons for that purpose which were exons (18),(19),(20) and (21) of the *EGFR* gene. After deparaffinization and DNA extraction from the tissues of patients with colorectal carcinoma, we used real-time PCR technique by using (Rotor gene) kit and we were run our samples with the control group of the same patients and internal control from the kit to compare if there was any mutation but there was not any mutation in those exons of our (30) samples of paraffin-embedded tissues (FFPE).

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Introduction

Colorectal cancer (CRC) is a common and lethal disease (1). And is one of the malignant tumors that are found most frequently around the world (2,3). The main cause for the high mortality rate is that the prognosis for progressed metastatic colon cancer is most unfavorable (4). Multiple genes in different genetic pathways are involved in the development of the pathogenesis of colorectal cancer. In recent years, the colorectal cancer molecular pathogenesis study has become very important (5). Many targeted agents have been developed. In particular, agents targeted at signal transduction for cell growth have been actively studied, and antitumor agents targeted at epidermal growth factor receptor (*EGFR*) are representative (6) *EGFR* is a cell-surface receptor for which expression increases in various malignant tumors, including CRC, and which affects cell growth and proliferation, metastasis, angiogenesis, and cell death through intracellular signal transduction (7,8) Signalling pathways that emerge from EGFR activation are critical in colon cancer (CC) biology. Its targeting with specific drugs has opened a new window in the treatment of this disease (2). (*EGFR*) is a tyrosine kinase receptor that shows overexpression in epithelial tumors and regulates important processes in tumorigenesis (9). Blocking *EGFR* activation would obviously represent an innovative key strategy in patient care because this therapeutic strategy impairs crucial cellular functions linked to proliferation and survival (10) Molecular markers that predict response to a specific therapy or treatment regimen are known as predictive biomarkers (11,12) In addition to molecular alterations of the *EGFR* gene, activation of *EGFR* downstream effectors can lead to tumor formation/progression. Specific alterations can impact prognosis and predict response to anti-*EGFR* therapy (13). In this research work, the *EGFR* gene expression rate in FFPE tissue of advanced colorectal cancer patients in Erbil was studied.

Materials and Methods

Among 134 individuals, we selected 30 CRC patients to investigate the prevalence of mutations in the Egfr gene. Of these patients, there were 10 women and 20 men. Using a Qiagen QIAamp, DNA was extracted from tissue samples that had been FFPE. The simple Egfr Mutation Analysis Kit for Real-Time PCR was utilized in order to carry out the mutation analysis (EntroGen). Table 1 provides a comprehensive overview of the demographic as well as clinical features of patients. Embedded tissues of colorectal cancer were used to select 30 out of 134 samples of archived formalin-fixed tissue for the purpose of RT-PCR molecular study.

DNA extraction

Genomic DNA was isolated from 10 µm-thick paraffin-embedded tissue sections. Sections were deparaffinated twice for 30 min in xylene, redehydrated in 100,80,60,40% ethanol for 10 sec., then add 200 ml Qiagen tissue lysis buffer (Qiaamp DNA extraction kit), transfer to eppendorf tubes and incubated with 40 µl protein-kinase and incubated overnight at 37°C add 20 ml protease K incubated for 1-2 h at 55°C after a total pro-k incubation DNA isolation proceeded as in the manufacture protocol.[22] DNA concen-

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tration was determined at 260 nm using the Nano-drop spectrum (thermos-fisher-USA).

EGFR mutation analysis using real-time PCR-based assay

Polymerase chain reaction PCR-based assay (Easy egfr kit) for identifying *EGFR* mutation, located in codons 18,19,20and 21. In a real-time or quantitative PCR, the product quantity is aforesaid during the reaction. The number of amplification cycles required to obtain a certain amount of PCR product is registered as the threshold cycle (Ct) (14–17). samples with ΔCt between 3.0 and 5.8, to confirm the mutation, must have a value of normalized fluorescence at the last cycle > 0.4. Compare the Ct and ΔCt values of the samples with those reported in Our table kit. The specified values are in the range and include extremes. The ΔCt values should be calculated with the following formula, taking care that the Ct value in Green/ Green2 for the mutation and the equivalent for the control assay belong to the same sample: T790M, S768I, L858R, L861Q, ex19del, ex20ins mix ΔCt = Ct Green mutation – Ct Green EGFR ctrl mix G719x mix ΔCt = Ct Green 2 mutation – Ct Green 2 *EGFR* ctrl mix. The assay was validated for analytical and diagnostic use and performed according to the manufacturer's instructions on a Real-Time PCR System (Roter- gene Q Qiagen.

Ethical consideration

The Ethical Committee at the College of Education, Salahaddin University has approved the study.

Statistical analysis

The statistical package for social science (SPSS, version 26) and Statgraphics were used for data entry and analysis. Descriptive statistical analysis (including frequency, percentage, mean, standard deviation, range, and ratio) was used to describe the data; and Inferential statistical analysis was used to determine the association between variables by using Fisher's exact tests with chi-square. The P-value is considered statistically significant if it's ≤ 0.05 which rejects the null hypothesis.

Results

The mean age of the study sample was 51.97 years, with SD ±15.962, the range of age is(=<20 to <61) years (Figure1).

The distribution of the sample according to sex was 64 % males and 36% females (Figure 2).

According to the data presented in Table 1, The sigmoid position exhibited the highest proportion of tumors, accounting for 38% of the total. This was followed by the proximal and mid-rectal locations, which accountined for 25% and 13%, respectively. Most (93%) tumors were adenocarcinoma- type. Concerning the size of tumors, the majority (22%) of tumors were less than 4 cm and 57% were more than 4 cm. Regarding metastasis, the table shows that 71% of tumors were positive in the metastasis. Same table show, that 69% of tumors were positive regarding the nodal state. In reference to pathological stages, table 1 shows that 57% of tumors were in grade III and 34% in grade II and according to this study 83% of them hadn't family history only 17% had a family history regarding colorectal carcinoma.

There wasn't any mutation in the *EGFR* gene of (30) CRC samples (Figure 3).

Gene expression analysis by real-time RT-PCR has been evaluated as a molecular determinant of the Mutation of the *EGFR* gene at four exons which were exons 18,19,20 and 21 in colorectal cancer. Thirty patients were included in the study. There was no association between colorectal cancer and gene expression of *EGFR*, which is in concordance with the Figure 3 presented here. The present data showed no significant correlation between *EGFR* expression in normal colon tissue compared to colon tumors. For supporting our study we presented the mutations of *EGFR* in internal control that contain within our kit. Figure 4 presents the mutations of the *EGFR* gene in internal control that shows the quality of our kit. The present study is the first evaluation of the possible relationship between *EGFR* gene expression in CRC in the Kurdistan region.

Discussion

The present investigation presentes findings on the incidence of CRC in the city of Erbil, including several factors associated with CRC cases.Specifically, our study focused on mutations happening at exons 18, 19, 20, and 21 of EGFR gene throught the period from 2020 to 2021. Furthermore, we used this data to make predictions regarding the occurence of new instances in the coming decade. Colorectal cancer was the third most common cancer among women and the fifth among men in Erbil governorate between 2013 and 2019. It represented the fifth most prevalent cancer in the Duhok governorate for both sexes. The reasons for the burden of colorectal cancer in the KRG are believed to reflect the changes in lifestyle and dietary factors, including smoking and obesity, which are associated with Westernized lifestyle factors 29. In 2018, colorectal cancer was the second most prevalent cancer among

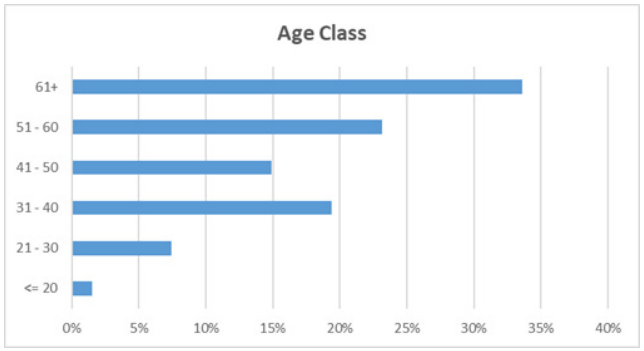

Figure 1. Percentage of different ages.

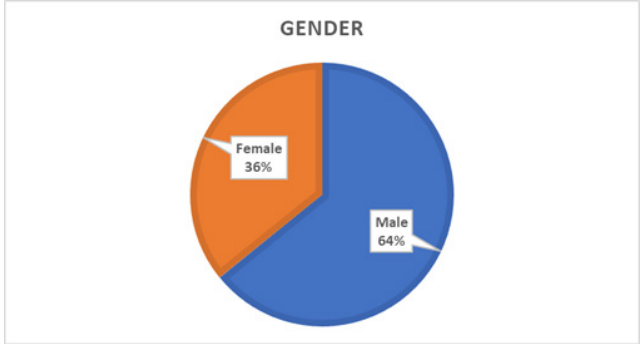

Figure 2. Percentage of both genders.

Table 1. Age, sex, clinical and histopathological features of CRC patients.

	Gender	
	N	%
Male	86	64%
Female	48	36%
Total	134	100%
	Age class	
	N	%
<= 20	2	1%
21 - 30	10	7%
31 - 40	26	19%
41 - 50	20	15%
51 - 60	31	23%
61+	45	34%
Total	134	100%
	Location of Tumor	
	N	%
Ascending	2	1%
Descending	12	9%
Lower Recta	10	7%
Mid rectal	18	13%
Proximal	34	25%
Sigmoid	51	38%
Transverce Colon	7	5%
Total	134	100.0
	Size of Tumor	
	N	%
<4	77	57%
4	28	21%
>4	29	22%
Total	134	100%
	Type of CRC	
	N	%
Adenocarcinoma	125	93%
Mucinous +signet ring	6	4%
Hyperchromatic	1	1%
Tubulo-villous adenoma	2	1%
Total	134	100%
	Grade	
	N	%
1	10	7%
2	46	34%
3	77	57%
4	1	1%
Total	134	100%
	Metastasis	
	N	%
Negative	39	29%
Positive	95	71%
Total	134	100%
	Family History	
	N	%
Negative	111	83%
Positive	23	17%
Total	134	100%
	Nodal State	
	N	%
Negative	41	31%
Positive	92	69%
Total	133	99%
System	1	1%
	134	100%

women worldwide, and the third among men; overall, CRC ranked third in prevalence, but second worldwide in mortality (18). The highest percentages of gender that diagnosed cases of CRC that reported in this study were in Erbil (61%) and the percentage of age was %34 had age 60 and more, this shows that the range was changed during 2020_2021 when we compared with the previous study. The reasons for the burden of colorectal cancer in

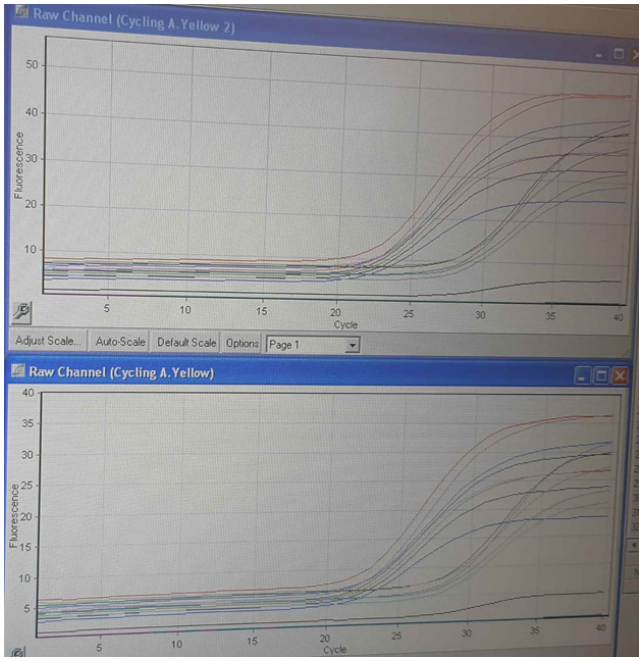

Figure 3. Graph of FAM baseline corrected normalized reporter of *EGFR* ex-19delin linear scale: No mutated samples (green and blue) have a Ct>31; wild-type samples (red) have a Ct<31.

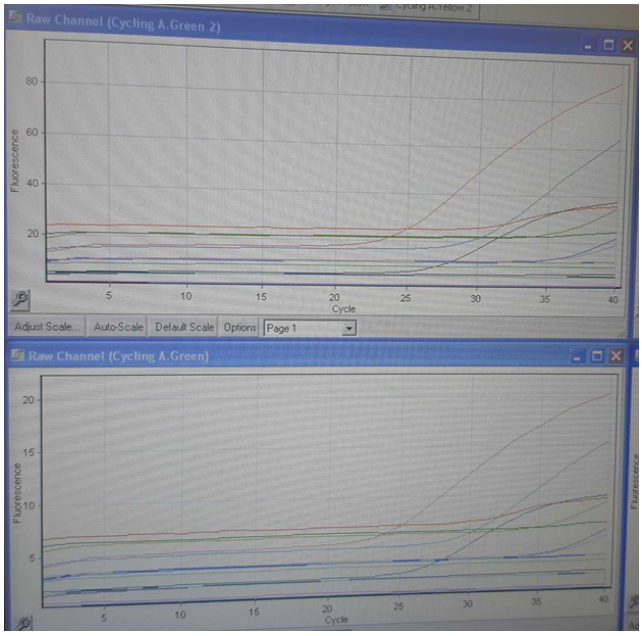

Figure 4. *EGFR* gene expression of internal control. Samples with ΔCt between 3.0 and 5.8 confirms the mutation. Red curve: Wild type sample; blue curve: Mutated sample

the KRG are believed to reflect the changes in lifestyle and dietary factors, including smoking and obesity, which are associated with Westernized lifestyle factors (19). In 2018, CRC was the second most prevalent cancer among women worldwide, and the third among men; overall, CRC ranked third in prevalence, but second worldwide in mortality (18). Likewise, in neighboring countries such as Iran, colorectal cancer ranked as the third most frequent cancer in 2015 (15). Thus far, previous studies focused on age, location of the tumor, size of the tumor, metastasis and type of CRC, nodal status, grade and family history. One of the most important risk factors for CRC incidence is the median age (51.97years) in which %34of cases of colorectal carcinomas age patients were 61years old or more than 61years old while only %1their age less than 20years old this was similar to the results of other studies that did on cancer in general In which conducted in Middle Eastern countries, such as Jordan (55 years 35 and 56 years 36) and Palestine (55 years 37). This could be at (19) attributable to the Kurdish population and other nations in the Middle East having younger populations than Western countries (14). However, the highest incidence was found in patients aged 70–79 years in some European countries, like the UK at 28.34% and Norway at 27.7% (15). The most frequent location of colon cancer was sigmoid Colon cancer in which %38 in which proximal comes after it which was %25 and only %1 of CRC patients have tumors in Ascending locations in the colon. According to the study that we did on our patients we found that %57 of CRC tumors size were <4 mm followed by %22 of them were their size of tumor >4mm and %21 of them were the tumor size =4mm. It is important to fully elucidate the biology of LN spread in CRC. Regarding with metastasis and nodal state we reached to the result that %71of patients had positive metastasis with 69% had positive nodal state while %29 had negative metastasis and %31 without nodal state. Accurate identification of lymph node (LN) involvement in patients with CRC is crucial for prognosis and treatment strategy decisions (16,20). Although several histopathologic findings, such as lymphatic invasion and tumor differentiation, are known to be predictors of LN metastasis, they are only available postoperatively. Preoperative knowledge of LN metastasis can provide valuable information for determining the need for adjuvant therapy and the adequacy of surgical resection, thus aiding in pretreatment decision-making (20,21). A family history of CRC is a known risk factor for CRC and encompasses both genetic and shared environmental risks. The prevalence of family history may be lower than the commonly cited 10% and confirms evidence for increasing levels of risk associated with increasing family history burden, The relative risk of developing CRC varied from 0.89 (for people with no family history) to nearly a 20-fold risk (for people with likely inherited syndromes), with risk levels in between, with increasing family history burden. The risk of CRC was higher when the relative was diagnosed at an earlier age. CRC risk also depended on the age of the person at risk: people with positive family history in their 30s or 40s demonstrated a higher relative risk compared to their age-matched peers than people with the same positive family history at an older age (22).There is evidence for a higher prevalence of adenomas and of multiple adenomas in people with a family history of CRC, but no evidence for differential adenoma location or adenoma progression by family history; depending on our study %83 of CRC patients had no family history related to their disease, while %17 of them had a family history of CRC so, for the foreseeable future family history may remain a valuable clinical tool for identifying individuals at increased risk of CRC.When we study the most prevalent colon cancer types we get that %93 of patients had adenocarcinoma type and %1 had Hyperchromatic and tubule_villous adenoma when we compared with other studies we find that Adenomatous polyps are the most common type of polyp in the colon, accounting for about 60% to 70% of all colonic polyps. Conventional-type adenomatous polyps can be classified as tubular, villous, or tubulovillous. Villous

adenomas are characterized by more than 75% villous features, whereas villous refers to finger-like or leaf-like epithelial projections. Tubulovillous adenomas have between 25% and 75% villous features. Less than 25% of villous features indicate a tubular adenoma. Adenomas are usually asymptomatic and found on routine CRC screening. Adenomas with villous features may be associated with a slight increase in the development of more advanced neoplasia or dysplasia compared to other types of adenomas (23-25).

EGFR assay might represent a suitable marker for the detection of circulating tumor cells in colon cancer patients. That mutation at exons 19, 20 and 21 of the *EGFR* gene of colon adenocarcinoma was significantly more frequently detected in CRC patients than in healthy controls supports the hypothesis that detection of mutation at those exons of *EGFR* gene are promising complementary marker for CRC staging and prediction of cancer progression and metastasis. RT-PCR assays with multiple tumor markers were shown to be superior in comparison to the assessment of single markers but due to their limited specificity; further data; investigation and clarification of the prognostic significance of genes and proteins implicated in metastatic process in CRC needs to be further investigated.

The therapy of colon and rectum tumors based on *EGFR* gene mutation remains under investigation, reserving huge potential for future applications and clinical interventions in conjunction with existing therapies. We expect, based on the previously exposed study, that the modulation of molecular markers, including the *EGFR* gene will stimulate the development of new therapeutic possibilities, making the treatment of colon and rectum tumors more effective in the Kurdistan region.

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None.

Interest conflict
The authors declare that they have no conflict of interest.

Source of funding
None.

Availability of data and materials:
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Study limitation
This study had some limitations. The sample size was small, and the data were analyzed retrospectively. The subjects were patients in a single medical institution, so the results may not apply to all colorectal cancer patients. Because the subjects did not undergo the same treatments, the correlation between the EGFR mutation and clinical outcomes could not be evaluated properly. However, considering that there have been no studies on the EGFR mutation in colorectal cancer patients, our study is meaningful as it investigates the incidence rate of the mutation.

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