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Novel Candidate Single Nucleotide Polymorphisms of ERCC2 Gene that Influence Colorectal Cancer Susceptibility

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Abstract

Colorectal cancer (CRC) is the most common gastrointestinal malignancy and one of the top ten common cancers worldwide with approximately 2 million cases. There are multiple risk factors that could lead to CRC emergence; of which are genetic polymorphisms. Excision repair cross-complementing group 2 (ERCC2) gene encodes for ERCC2 enzyme which plays a crucial role in maintaining genomic integrity by removing DNA adducts. Several studies suggested that there could be a link between genetic polymorphisms of ERCC2 gene and the risk of CRC development. Hence the present study aims to validate the relationship between the following ERCC2 single nucleotide polymorphisms (rs13181, rs149943175, rs530662943, and rs1799790) and CRC susceptibility. A total of 121 participants were enrolled in this case control study; 72 CRC patients and 49 apparently healthy individuals. CRC patients aged 56.34 ±11.89 years and 41 (56.9%) were males while control group were 53.20 ± 17.33 years and 26 (53.1%) of them are males. Genotyping was performed using polymerase chain reaction (PCR) followed by Sanger sequencing then the association between genetic polymorphisms and CRC susceptibility was examined. GA genotype and A allele of rs149943175 were associated with lower risk of CRC development [OR 95% (CI)= 0.3 (0.1-0.88); P=0.02 and 0.4 (0.1-0.9); P=0.03 respectively]. However, GA genotype and A allele carriers of rs530662943 had significantly increased risk compared to GG genotype and G allele respectively [OR 95%(CI)= 5.17 (1.1-24.0); P=0.03 and 4.76 (1.0-21.6); P=0.04 respectively]. Additional stratified analyses showed that carriers of heterozygous genotype of rs149943175 who non-smokers, females or BMI figures less than 25 are less likely to develop CRC compared to wild genotype carriers. Taken together, genetic polymorphisms of ERCC2 modulate the susceptibility of CRC malignancy.
Keywords: Colorectal cancer, ERCC2 gene, NER pathway, XPD

تقييم تعدد الاشكال الوراثية الجديدة في جين ERCC2 المرشحة لقابلية الاصابة بسرطان القولون والمستقيم

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الخلاصة

سرطان القولون والمستقيم هو أكثر الأورام الخبيثة المعدية المعوية شيوعا وواحد من أكثر عشرة سرطانات شائعة في جميع أنحاء العالم مع ما يقرب من ٢ مليون حالة. هناك العديد من عوامل الخطر التي يمكن أن تؤدي إلى ظهور سرطان القولون والمستقيم منها تعدد الأشكال الوراثية. يشفر جين المجموعة ٢ المتممة لإصلاح الاستئصال لإنزيم ERCC2 الذي يلعب دورا حاسما في الحفاظ على السلامة الجينومية عن طريق إزالة اجزاء الحمض النووي التالفة. اقترحت العديد من الدراسات أنه يمكن أن يكون هناك صلة بين تعدد الأشكال الجينية لجين ERCC2 وخطر تطور مرض السرطان. ومن ثم تهدف الدراسة الحالية إلى التحقق من صحة العلاقة بين تعدد أشكال النوكليوتيدات المفردة ERCC2 (rs13181, rs149943175, rs530662943, and rs1799790) وقابلية ظهور سرطان القولون والمستقيم. لقد شارك في هذه الدراسة ١٢١ فرد حيث شكل ٧٢ منهم مرضى السرطان و٤٩ هم الاشخاص الاصحاء. تم اجراء التنميط الجيني باستخدام تفاعل البلويمريز المتسلسل ثم تم فحص الارتباط بين تعدد الاشكال الوراثية وقابلية ظهور المرض.

النتائج ارتباط النمط الجيني GA والاليل A لrs149943175 بانخفاض خطر ظهور سرطان القولون والمستقيم. اما النمط الجيني GA والاليل A فقد ارتبطا بزياده خطر الاصابة بسرطان القولون والمستقيم. اظهرت التحليلات الاضافية ان حاملي النمط الجيني GA لrs149943175 من هم من غير المدخنين, الاناث ولديهم مؤشر كتله الجسم اقل من ٢٥ لديهم احتمالية اقل للاصابة بالمرض. بالمجمل ان تعدد الاشكال الوراثية في جين ERCC2 تؤثر على احتمالية ظهور سرطان القولون والمستقيم

الكلمات المفتاحية: سرطان القولون والمستقيم, ERCC2 , مسار NER , جفاف الجلد المصطبغ مجموعة د

Introduction

Cancer is a group of various diseases characterized by uncontrolled cellular proliferation, local tissue invasion, and distant metastases [(1)]. It is one of the top causes of mortality worldwide, with about 10 million deaths in 2020 [(2)]. Of the most widespread malignancies is colorectal cancer (CRC) which has been registered as the third most common cancer globally where 2 million cases were diagnosed in the year 2020 [(3)]. Likewise, recent statistics in Iraq revealed that it is the third most common cancer with 2,328 new cases in the year 2019 [(4)]. CRC is a multistep process where it starts with an abnormal growth of polyp that originates from the inmost layer of the colon. A polyp may take 10 to 15 years to develop into a malignant growth [(5, 6)]. There are numerous risk factors; most of which co-occur and interact to cause CRC malignancies such as increased age, male gender, smoking, alcohol, obesity and many other environmental as well as lifestyle related factors[(7, 8)]. Nevertheless, one of the most significant CRC risk factors is genetic polymorphisms [(9)]. In CRC pathogenesis, three main mechanisms that influence genes functions have been identified: microsatellite instability, chromosomal instability and Cytosine guanine (CpG) island methylator phenotype [(10, 11)]. In addition, mutations in genes related to DNA repair pathway especially nucleotide repair pathway (NER) may lead to altered DNA repair function and reduced genomic integrity which modulate individual's susceptibility to develop cancers as NER pathway is responsible for reversing DNA damage by removing bulky DNA adducts caused by various factors[(12, 13)]. Excision Repair Cross-Complementing Group 2 (ERCC2) gene which is also known as Xeroderma Pigmentosum group D (XPD) is located at chromosome 19q13.3 and includes 23 exons which span around ~54.3 kb in length; the protein produced by this gene; ERCC2; belongs to ATP-dependent 5′-3′ superfamily 2 helicases which plays a pivotal role in NER pathway and have been studied extensively in many types of malignancies of which is CRC[(14, 15)]. Different single nucleotide polymorphisms (SNPs) of ERCC2 have been correlated with CRC risk as researchers postulated that these polymorphisms modulate protein functions that consequently impact genetic stability and thus the onset of cancer[(16)]. The most frequently investigated SNPs are rs13181 (Lys751Gln), rs1799793 (Asp312Asn), and rs238406 (Arg156Arg).

A meta-analysis that was published in 2022 showed that rs1799793 was significantly associated with CRC risk while rs13181 was linked to CRC risk only among Asians [(17)]. Salimzadeh *et al* investigated the impact of rs13181 and rs238406 on CRC susceptibility and concluded that both SNPs are significantly associated with increasing the risk of CRC emergence [(18)]. Research was conducted among polish population showed that AA genotype of both rs238406 and rs1799793 were related with elevated CRC risk. Nonetheless, CC genotype was protective against CRC development [(19)]. Balkan *et al* revealed a significant association of GG and TG genotype of rs13181 with increased risk of developing CRC[(20)]

We have recently investigated the impact of rs1799793, and rs238406 polymorphisms on CRC susceptibility among Iraqi population and lack of association was concluded [(21)]. Therefore, the aim of this study is to investigate the possible role of the following SNPs (rs13181, rs149943175, rs530662943, and rs1799790) in the development of CRC among Iraqi cancer patients. Recently, there have been various efforts in Iraq to identify genetic markers for many malignancies as well as serious diseases whose treatment may results in quality-of-life limitation [(22-25)]. Identifying potential biomarkers for individuals at risk of CRC will help with the implementation of new early detection methods and preventative lifestyle measures to lower the incidence rate of CRC in the target population.

Materials and Methods

Study population

This is a case control study which involved two groups; the first group includes 72 CRC patients whose diagnosis was confirmed by colonoscopy and histopathological biopsy [(26)]. They were recruited from Oncology teaching hospital/Medical city/Baghdad; their CRC stage was from II to IV. The other group is 49 apparently healthy individuals considered eligible as a control group if they were age, gender, BMI (Body Mass Index), and smoking status matched. Both groups were over the age of 18 years and the control group were free from family history of cancer. Recruitment of study participants was done between February to December 2022. The research was approved by the ethics committee in Baghdad University/ College of Pharmacy (approval number: RECAUBCP26102021B) in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national). Informed consent was obtained from all participants before inclusion into the study. CRC was staged according to TNM (Tumor Node Metastasis) classification system [(27)]. using information from patients' files that was also

used to collect clinical data. Tumor differentiation information was obtained from pathological reports. Regarding smoking status of participants, were acquired from the study questionnaire and classified as ever versus never. participants were considered ever smokers if they are currently smoking or previously smoked before the run of the study. Never smokers were defined as subjects who had never smoked in their life.

DNA extraction and Genotyping

Three milliliters of peripheral venous blood were collected from patients and controls in K3EDTA coated tubes and DNA extraction was performed by ReliaPrep™ Blood gDNA Miniprep System (Promega, USA) according to manufacturer's instructions. DNA sequence to be amplified was obtained from the National Center for Biotechnology Information (NCBI) GenBank database.

ERCC2 primers were designed using Premier 3 software (version 0.4.0). The DNA template was amplified using the primer pairs (forward and reverse) at annealing temperatures of 55, 58, 60, 63, and 65°C in order to find the ideal annealing temperature for primers. The optimum annealing temperature was 60 ^{0}C and Polymerase chain reaction (PCR) amplicon length was 996 BP. Regarding primer sequence; forward primer: TGTAAAACGACGGCCAGTCCCTCAGCAAAG AGAAGTTTA while reverse primer sequence: CAGGAAACAGCTATGACCAGGACAGGAGC AAAGATG. PCR technique was conducted using a thermal cycler (Thermo Fisher Scientific, USA). The PCR amplicon was sent for Sanger sequencing using automated DNA sequencer by Macrogen Corporation – Korea then the results were retrieved by email and analyzed using geneious software (V 2021.1.1).

Statistical analysis

Data were analyzed using SPSS software version 26 (SPSS® Inc, Chicago, USA). Categorical Data were expressed as count and percentage whilst continuous data were expressed as mean ± SD. Genotype distribution within the groups of the cases and controls was compared by Hardy-Weinberg equilibrium (HWE) using the chi-square test. Demographic and clinical data were compared with Student T test, chi square or fisher exact test as appropriate. The strength of association was assessed by calculating odds ratio (OR) and 95% confidence interval (95% CI). p value < 0.05 was considered to be statistically significant.

Results

Patients' characteristics

The current study included a total of 121 subjects (72 CRC patients and 49 apparently healthy subjects). The average age of patients and healthy participants was (56.34 ±11.89 years and 53.20 ± 17.33 years) respectively. There was no statically significant difference in mean age distributions between disease and control groups (p value=0.33). Male to female ratio in CRC cases was ~ 1.3 (41 male vs 31 female); Controls were selected in the similar ratio to match the gender (p value =0.67). Same applies to smoking status where ratio of smokers to non-smokers in patients was 0.8. On the other hand, the ratio in the control group was 0.88 (p value= 0.78). Patients' clinical data including tumor location, CRC stage and tumor differentiation are illustrated in (Table 1).

Table 1. Characteristics of patients with colorectal cancer and controls.

Parameters	Cases N (%)	Control N (%)	P value
Age (years) (mean ± SD)	56.34 ±11.89	53.20 ± 17.33	0.33[a]
Gender			
Male	41 (56.9%)	26 (53.1%)	0.67[b]
Female	31 (43.1%)	23 (46.9%)	
Smoking			
Yes	32 (44.4%)	23 (46.9%)	0.78[b]
No	40 (55.6%)	26 (53.1%)	
BMI [c] (Kg/m^2) (mean ± SD)	26.14±4.04	27.4±4.7	0.17[a]

[a] independent sample t test was used, [b] chi square was used, [c] BMI: Body Mass Index.

Table 2. Disease characteristics of CRC patients

Variables	N (%)
Tumor location	
Colon	51 (70.8%)
rectum	21 (29.2%)

Continued Table 2.

Tumor differentiation	
Well	13 (18.1%)
Moderate	44 (61.1%)
poor	15 (20.8 %)
Stage	
II	31 (43.1%)
III	29 (40.3%)
IV	12 (16.6%)
Tumor invasion	
T1 and T2	12 (16.7%)
T3 and T4	60 (83.3%)
Lymph node involvement	
Yes	29 (40.3%)
No	43 (59.7%)
Metastasis	
Yes	12 (16.7%)
No	60 (83.3%)

Polymorphism distribution and its correlation with CRC risk

Distribution of genotypes and alleles of patients and controls are given in (Table 2). The distributions of the genotypes and alleles of the four SNPs were in Hardy–Weinberg equilibrium except for patients' group of rs13181 and rs1799790. Frequencies of rs13181 and rs1799790 minor genotypes are found to be comparable in both CRC patients and healthy controls where P values were > 0.05 (Table 3).

Regarding rs149943175, it was found that carriers of GA genotype and A allele are less susceptible to develop malignancy [OR (95%CI) = 0.3 (0.1-0.88); 0.4 (0.1-0.9); P=0.02 and 0.03 respectively]. On the other hand, heterozygous genotype of rs530662943 has significantly increased risk by 5.17 times compared to homozygous dominant genotype [OR (95%) =5.17 (1.1-24.0); P=0.03]. Furthermore, A allele carriers are 4.76 more likely to have CRC Cancer [OR (95%CI) = 4.76 (1.0-21.6); P=0.04].

Table 3. Genotypes and allele frequencies for ERCC2 SNPs in colorectal cancer patients and controls.

Genotype/allele	controls N=49 (%)	patients N=72 (%)	P value	OR 95% CI
rs149943175				
GG	32(65.31%)	60(83.33%)	**0.02***	2.6 (1.1-6.3)
GA	17(34.69%)	12(16.67%)	**0.02***	0.3 (0.1-0.88)
G	81(82.65%)	32(91.67%)	**0.03***	2.3 (1.04-5.20)
A [b]	17(17.35%)	12(8.33%)	**0.03***	0.4 (0.1-0.9)
rs13181				
TT	17(34.69%)	31(43.06%)	0.4	
TG	21(42.86%)	24(33.33%)	0.2	
GG[a]	11(22.45%)	17(23.61%)	0.7	
T	55(56.12%)	86(59.72%)	0.5	
G[b]	43(43.88%)	58(40.28%)	0.5	
rs530662943				
GG	47(95.92%)	59(81.94%)	**0.03***	0.19 (0.03-0.8)
GA	2(4.08%)	13(18.06%)	**0.03***	5.17 (1.1-24.0)
G	96(97.96%)	131(90.97%)	**0.04 ***	0.21 (0.03-0.8)
A [b]	2(2.04%)	13(9.03%)	**0.04***	4.76 (1.0-21.6)
rs1799790				
CC	18(36.73%)	35(48.61%)	0.2	
CG	25(51.02%)	24(33.33%)	0.08	
GG[a]	6(12.24%)	13(18.06%)	0.8	
C	61(62.24%)	94(65.28%	0.6	
G [b]	37(37.76%)	50(34.72%)		

*Statically significant, P value calculated using chi square or fisher exact test as appropriate, [a] minor genotype, [b] minor allele

Stratification analysis by demographics for ERCC2 SNPs and CRC risk

The possible association between ERCC2 genetic polymorphisms and CRC susceptibility was further examined through stratification by age, gender, smoking status, and BMI. The analysis showed that non-smoker carriers of GA genotype of rs149943175 are less prone to develop CRC compared to GG genotype carriers [OR (95%CI) = 0.15 (0.049-0.498); P=0.001]. Moreover, reduced risk of CRC associated to the heterozygous genotype was observed in female patients [OR (95%CI) = 0.18 (0.05-0.62); P=0.007] but not in males. Concerning BMI, normal weight patients with GA genotype are less susceptible to having CRC [OR (95%CI) = 0.3 (0.09-0.96); P=0.039]. regarding age; there was no difference in genotype distribution between individuals who are aged 50 years or younger and older participants aged more than 50 years; Data are illustrated in table 3. Other SNPs had no association with CRC risk stratified by age, gender, smoking status, or BMI. (supplementary).

Table 4. Association between rs149943175 and risk of CRC stratified by demographic characteristics.

SNPs		genotype	CRC	Control	P value	OR (95% CI)
Smoking status	Yes	GG	26	20	0.72[a]	1.5 (0.3-6.9)
		GA	6	3		
	No	GG	33	12	**0.001[*b]**	**0.15 (0.05-0.5)**
		GA	6	14		
gender	male	GG	34	21	1[b]	0.86 (0.2-3.1)
		GA	7	5		
	Female	GG	26	11	**0.007[*b]**	**0.18 (0.05-0.62)**
		GA	5	12		
Age (years)	≤ 50	GG	17	13	0.18[a]	0.33 (0.08-1.35)
		GA	4	9		
	>50	GG	42	19	0.23[b]	0.45 (0.15-1.4)
		GA	8	8		
BMI (Kg/m^2)	Normal [c]	GG	25	15	**0.039[*b]**	**0.3 (0.09-0.96)**
		GA	6	12		
	Overweight [d]	GG	21	10	0.64[a]	0.47 (0.08-2.8)
		GA	3	3		

[*]statically significant, [a] Fisher exact test used, [b] chi square test used, [C] BMI ≤24.9, [d] BMI≥ 25, BMI: Body Mass Index

Discussion

In the present research we conducted a case–control study consisting of 72 confirmed CRC cases and 49 controls to investigate the relationship between *ERCC2* genetic polymorphisms rs13181, rs149943175, rs530662943, and rs1799790) and the risk of CRC in a sample of Iraqi population. rs13181 represents non-synonymous mutation corresponding to amino acid change from lysine to glutamine at codon 751 which results in conformational change at the C-terminal of the protein that represents area of contact between ERCC2 protein and helicase activator and has been extensively studied with a wide range of diseases and malignancies including CRC [14] [28]. rs149943175 and rs530662943 have never been studied previously while rs1799790 have only been studied with lung cancer [29]. Very few reports have been published related to the relationship of *ERCC2* gene variants and malignancy risk. Jasiem *et al* examined the relationship of rs13181 (Lys751Gln) with susceptibility to develop acute myeloid leukemia (AML) among Iraqi population and found that carriers of GG genotype have higher risk of developing AML while T allele may be protective against AML [30]. On the other hand, Al-ward et al assessed the impact of rs13181 on lung cancer risk among Iraqi individuals and revealed that carriers of heterozygous genotype and C allele are more prone to develop lung cancer [31]. The current study showed that no association was found between rs13181 polymorphism and risk of developing CRC. Consistent to this research findings are reported in Turkish, Mexican, Algerian, Polish, and Chinese population [32-36]. Contrary to the present research results, two studies revealed significant association with both variant homozygous and heterozygous genotype of Lys751Gln polymorphism among Turkish and Pakistani population [20, 37]. In addition, a study revealed that C allele carriers have increased susceptibility by 1.4 time compared to AA genotype among Swedish population [18]. However, research conducted on a sample of Chinese population concluded that A allele carriers are the ones who are more prone to CRC development [38]. Another study was also done on Chinese population with lynch syndrome found that carriers of heterozygote are at increased risk than homozygous wild type [39]. These contradictory results can be explained by differences

in genotype and allele distributions among diverse ethnic populations. Moreover, environmental risk factors and dietary lifestyle could influence epigenetic modifications [5]. Although in this study no statically significant association between being smoker, carrying rs13181 variant and elevating the risk of CRC was found, other researchers observed an association between being smoker, having a variant genotype or allele and CRC emergence [37]. Concerning rs149943175, this study concluded that carriers of GA genotype and A allele are less susceptible to develop malignancy indicating that being heterozygous at rs149943175 possibly offers better DNA repair capacity and hence be protective against CRC. These results was also confirmed when data stratified by smoking status, BMI and gender; gender disparities in CRC risk are possibly owing to estrogen's protective action that causes stimulation of apoptosis and prevention of cell proliferation [40]. Concerning BMI, this study revealed that GA genotype whose BMI less than 25 kg/m^2 are less prone to have malignancy. Obesity is marked as a risk factor for CRC development; A meta-analysis found that CRC risk rise by18% for every 5 units increment in BMI with stronger associations has been in men than women, and for colon in comparison to rectal cancers [14]. On the other hand, the heterozygous genotype of rs530662943 has significantly raised risk by 5.17 times compared to homozygous dominant. However, A allele carriers are 4.76 more likely to have CRC Cancer. rs149943175 and rs530662943 are 3 prime Untranslated Region variants (3′UTR); they have never been before. The 3′UTR is sited downstream of the coding sequence, and its major role in regulatory processes that includes RNA stability, mRNA translation and localization. The 3′ UTR is characterized by binding sites for RNA binding proteins and miRNAs, and therefore any differences in the 3′UTR length and sequence may result in binding site changing for miRNAs and RBPs, ultimately causing alteration in gene expression [41]. Concerning rs1799790, it did not reach statistical significance in the present investigation. It is an intron variant that has been investigated previously with risk of smoking related lung cancer and researchers found lack of association between rs1799790 genotypes and susceptibility to lung cancer [29].

Although this study produced some significant findings, there were also a few limitations. The small sample size did not allow the study to have adequate power. However, studies of a similar nature had recruited sample sizes close to or even smaller than ours. Additionally, there are other genes of NER pathway (ERCC1, ERCC3, XRCC1,.) that need to be studied in order to form a complete picture of their interaction together. The possibility that the associations found in the present research happened by chance cannot be excluded, and additional verification in larger samples and different regional populations are needed to confirm these results.

Conclusion

This research was designed to elucidate the impact of selected polymorphisms in DNA repair gene and risk of CRC pathogenesis. The findings show an increased risk for CRC in individuals with rs530662943 GA genotype and A allele. On the other hand, rs149943175 heterozygous genotype and variant allele offers protection against CRC suggesting NER pathway modulate the CRC risk in Iraqi population. Rs13181 and rs1799790 did not reach statistical significance. Nevertheless, these associations require investigations in other populations and functional characterization of the variant consequences in CRC pathogenesis.

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Conflicts of Interest

The authors declare no conflict of interest for this article.

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Ethics Statements

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Baghdad University/ College of Pharmacy (approval number: RECAUBCP26102021B on 26-10-2021)

Author Contribution

Study design (RF, ESS, AZA); conduct of study (RF); collection of data (RF); analysis, interpretation, and management of data (RF, ESS, AZA); preparation of manuscript (RF); intellectual content review (ESS, AZA); and approval of final manuscript draft (RF, ESS, AZA).

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