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RESEARCH ARTICLE

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Association between nucleotide excision repair gene polymorphism and colorectal cancer risk

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Abstract

Background: The nucleotide excision repair system removes a wide variety of DNA lesions from the human genome, and plays an important role in maintaining genomic stability. Single nucleotide polymorphisms (SNPs) in nucleotide excision repair are associated with the various forms of tumor susceptibility. However, the relationship between NER polymorphism and colorectal cancer is not clear.

Methods: In this study, three candidate SNPs including ERCC4 (rs6498486), ERCC1 (rs3212986), and ERCC5 (rs17655) were analyzed in 1101colorectal cancer patients and 1175 healthy control patients from Jiangsu province (China). Then, we performed Immunohistochemistry, qPCR, and luciferase assay to determine the potential mechanisms.

Results: The ERCC4 rs6498486 AC/CC genotypes show lower susceptibility to CRC than those carrying rs6498486 AA (Adjusted OR = 0.82, 95% CI = 0.69-0.97). However, we did not observe any association between the colorectal cancer risk and the rs3212986(ERCC1) and rs17655(ERCC5) polymorphisms. Immunohistochemistry, qPCR, and luciferase assay revealed that rs6498486 A > C polymorphism in the ERCC4 promoter region could lessen the expression level of ERCC4 by impacting the binding ability of the transcription factor NF-kB, thereby affecting the transcription activity of the ERCC4 gene and decreased ERCC4 gene expression.

Conclusion: In brief, our finding demonstrated that ERCC4 rs6498486 serves as a potential biomarker of CRC susceptibility for the development of colorectal cancer.

KEYWORDS
colorectal cancer, ERCC4, polymorphism, rs6498486

1 | INTRODUCTION

Colorectal cancer is the third most common cancer and the fourth leading cause of cancer-related mortality, including more than 1.2 million new cases and 0.6 million deaths each year.[1] Although the exact pathogenesis of colorectal cancer is still unknown, environmental factors, diet, smoking, drinking, and obesity are believed to contribute to its onset.[2] However, not all individuals exposed to these risk factors can develop CRC, which suggests that some other factors, perhaps including gene polymorphisms, may contribute to the variation in inter-individual susceptibility to CRC.[3] In addition, some studies have shown

Abbreviations: CI, confidence interval; CRC, colorectal cancer; ERCC4, excision repair cross-complementing 4; HWE, Hardy-Weinberg equilibrium; NER, nucleotide excision repair; SNP, single nucleotide polymorphism.

Zhang and Wu contributed equally to this work.

that individual predispositions for developing this cancer may depend on genetic changes, including changes in genes involved in the process of DNA repair, which is responsible for dealing with DNA damages.[4,5]

DNA repair systems play a crucial role in maintaining the genome stability, which include nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), and double-strand break repair (DSBR).[6,7] Typically, NER is listed as biochemical tool to deal with UV-induced damage such as cyclobutane-pyrimidine dimers and 6-4 pyrimidine-pyrimidone photoproduct.[8] NER process had been reported to contain three steps, including damage recognition, unwinding of DNA and removal of the damaged fragment, and DNA synthesis.[9,10] Several types of genes are known to be involved in NER, including ERCC1, ERCC4, and ERCC5.[11,12] Genetic variations in ERCC1, ERCC4, and ERCC5 gene affect repair of bulky DNA lesions, maintenance of genomic stability, and thus affect cancer risk.[13,14]

NER gene polymorphisms have been extensively studied in terms of their associations with cancer risk as well as clinical outcomes in specific cancer types.[15-17] The previous study had shown that polymorphisms in DNA repair genes involved in nuclear excision repair (NER) could alter the efficacy of DNA repair and thus influence individual susceptibility to colorectal cancer.[18,19] Although many efforts have been made to explore the role of ERCC1, ERCC4, and ERCC5 polymorphisms in the NER pathway in the development of colorectal cancer, but it is still not identified the underling functional mechanisms between these polymorphisms and the colorectal cancer risk.[20,21]

In our study, we performed a case-control study on 1101 colorectal cancer patients and 1175 matched healthy controls to determine the association between DNA repair gene (ERCC1, ERCC4, and ERCC5) polymorphisms and the risk of colorectal cancer and further reveal the functional mechanisms of CRC risk.

2 | MATERIALS AND METHODS

2.1 | Study subjects

In our study, we recruited a total of 1101 newly diagnosed and histologically confirmed colorectal cancer patients, in the Jiangsu province (China) between January 2014 and October 2017. Healthy controls (1175) were randomly selected from the same hospital for physical examination. The controls were genetically unrelated to the colorectal cancer cases. The pathologic stage of colorectal cancer was assessed using the Sixth Edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual. All participants signed an informed consent form, and all activities on human subjects were carried out under full compliance with the Helsinki Declaration. The study was approved by the Ethics Committee of Southeast University Affiliated Zhongda Hospital (Nanjing, China).

2.2 | DNA extraction and genotyping

Genomic DNA was extracted from whole-blood samples using the RelaxGene Blood DNA System (Tiangen Biotech). The genotypes of the rs6498486, rs3212986, and rs17655 polymorphisms were determined by TaqMan allelic identification assay equipped with A Quant Studio 6 Flex system (Applied Biosystems, Life Technologies). The loading wells without DNA were used as negative controls. 10% samples were randomly selected for verification. The accordance rate was 100%.

2.3 | Real-time PCR Assay

Total RNA was isolated from colorectal cancer tissues using Trizol reagent (Invitrogen). SYBR Green Realtime PCR (Toyobo) assay was determined by Quant Studio 6 Flex system (Applied Biosystems, Life Technologies). β-actin was chosen as internal control. Primer sequences were 5'-TCCTCAGTTGAACCTCCGTAT-3' (forward) and 5'-ACCCCTCACTATCATCCATCC-3' (reverse) for ERCC4 and 5'-ATCCGCAAAGACCTGT-3' and 5'-GGGTGTAACGCAACTAAG-3' for β-actin. Each reaction was done in triplicate.

2.4 | Immunohistochemistry

Paraffin-embedded sections fixed by formalin were dewaxed in xylene and then hydrated with ethanol gradient. Endogenous peroxidase was blocked with methanol containing 0.3% hydrogen peroxide for 30 minutes. To retrieve antigenicity, sections were boiled in 10 mmol/L citrate buffer (pH 5.8) for 30 minutes in a microwave (800 W). Next, the slices were placed in 1× CB buffer to room temperature, then transferred to TBS solution, and soaked in TBS solution for 5 minutes. Afterward, the sections were incubated at 4°C for 48 hours with primary antibodies specific for ERCC4 diluted at 1:100. After 2 days, sections were rinsed with fresh TBS solution and incubated at room temperature with horseradish peroxidase-linked secondary antibodies for 30 minutes. Finally, the sections were stained with 3,30-diaminobenzidine (DAB) substrate and counterstained with hematoxylin. Images were recorded using a microscope. ERCC4 IHC was classified using the following scoring scheme proposed: negative or weak, ≤3; moderate, >3 but ≤6; and strong, >6.

2.5 | Cell culture

The human colorectal cancer cell line (RKO and HT-29) and HEK293T were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Sigma, St. Louis), penicillin and streptomycin in 5% CO_2 at 37°C. The cells were periodically detected and verified to be mycoplasma-free. These three cell lines were detected by short tandem repeat analysis and used within 6 months.

2.6 | Luciferase assay

293T, RKO, and HT-29 cells were seeded into 6-well plates and cultured for 16-24 h until grown to 50%-60% confluence. The ERCC4 promoter sequence containing rs6498486 A or C allele was constructed into pGL3-basic vector. They were then co-transfected with a firefly luciferase expressing plasmid (2 μg) and internal control

TABLE 1 Demographic characteristics of CRC patients and controls

Variables	Case (N = 1101)		Control (N = 1175)		*P*
	n	%	n	%	
Age, y (mean ± SD)	56.39 ± 0.3		56.12 ± 0.4		0.586
Gender					
Male	685	62.3	686	58.4	0.058
Female	415	37.7	489	41.6	
Location					
Colon	540	49.1			
Rectum	561	51.0			
Grade					
Low	343	32.2			
Intermediate	517	48.6			
High	204	19.2			
Depth of invasion					
T1	50	4.7			
T2	186	17.3			
T3	91	8.5			
T4	748	69.6			
Lymph node metastasis					
N0	594	54.0			
N1	507	46.1			
Distant metastasis					
M0	669	60.8			
M1	432	39.2			
TNM					
I	109	9.9			
II	263	23.9			
III	297	27.0			
IV	432	39.2			

vector pRL-SV40 (2 μg) as an internal control, using Lipofectamine 2000 reagent (Invitrogen), in accordance with the manufacturer's protocol. After 48 hours, we measured luciferase activity using a Dual Luciferase Reporter Assay (Promega). The relative luciferase activity was estimated by normalizing firefly luciferase activity to that of Renilla for each assay.

2.7 | Statistical analysis

The Hardy-Weinberg equilibrium (HWE) was estimated in controls using the goodness-of-fit chi-square test (χ^2 test). Both t test and $\chi 2$ test were applied to describe the frequency distribution of the demographic characteristic and genotype results of the SNPs between the cases and controls. Genotype and allele frequency differences between cases and controls were evaluated using logistic regression analysis, with odds ratios (OR) and 95% confidence intervals (95% CI), adjusted for age and sex. All statistical analyses were conducted using SAS. *P* value of less than 0.05 was considered statistically significant.

3 | RESULT

3.1 | Demographic and Characteristic of the study population

The control information on the subjects has been shown previously.[22] The demographic and clinicopathological characteristics of the colorectal cancer cases and healthy controls are exhibited in Table 1. In our study, we recruited 1101 cases and 1175 controls. And the frequencies of age and gender between the cases and controls were comparable ($P > 0.05$ for age and gender). The clinical pathologic data of the case patients including location, grade, depth of invasion, lymph node metastasis, distant metastasis, and TNM were also summarized in Table 1. In the case group, 540 patients were colon cancer (49.1%) and 561 patients were rectum cancer (51.0%). More intermediate grade (48.6%) was observed than low grade and high grade. 54.0% of the patient were found with negative lymph node metastasis and 39.2% presented the distant metastasis. Meanwhile, the frequencies of depth of invasion

were 4.7% (T1), 17.3% (T2), 8.5% (T3), and 69.6% (T4), respectively. 9.9% of patients were in stage I, 23.9% in stage II, 27.0% in stage III, and 39.2% in stage IV.

3.2 | Relationship between the selected polymorphisms and colorectal cancer susceptibility

The association between the four SNPs and colorectal cancer risk is shown in Table 2. The genotype distributions in control were in accordance with Hardy-Weinberg equilibrium (P > 0.05). The rs6498486 AC/CC genotypes were associated with decreased colorectal cancer risk (adjusted OR = 0.82, 95% CI = 0.69-0.97). However, no significant differences were observed between the risk of CRC and the rs3212986 and rs17655 polymorphisms.

3.3 | Stratified analysis of rs6498486 polymorphism and colorectal cancer risk

Stratified analysis was performed to evaluate the association between the rs6498486 polymorphism and colorectal cancer risk. As shown in Table 3, the rs6498486 AC/CC genotype suffered a significantly higher risk in the subgroups of Male (adjusted OR = 0.76, 95% CI = 0.61-0.95), negative lymph node metastasis (adjusted OR = 0.80, 95% CI = 0.66-0.99), positive distant metastasis (95% CI = 0.73, 95% CI = 0.58- 0.92), and TNM IV (adjusted OR = 0.73, adjusted OR = 0.58-0.92).

3.4 | rs6498486 CC genotype decreased the ERCC4 expression levels by reducing the ERCC4 transcriptional activity

On the basis of our previous findings, we found that rs6498486 A > C polymorphism decreased the risk of colorectal cancer. Because rs6498486 polymorphism is located in the ERCC4 promoter region, we speculate that the ERCC4 rs6498486 polymorphism may affect the binding of transcription factors to promoter regions. Therefore, we analyzed the ERCC4 rs6498486 A > C polymorphism promoter region by using a bioinformatics algorithm (AliBaba2). And we found that the rs6498486 A > C polymorphism might alter the binding ability of NF-kB to the rs6498486 mutation region (Figure 1D).

TABLE 2 Association between ERCC4, ERCC1, and ERCC5 polymorphisms and colorectal cancer risk

	Cases		Controls			
Genotype	n	%	n	%	*P*	Adjusted OR (95% CI) [a]
ERCC4 rs6498486						
AA	678	61.9	665	57.1	**0.043**	1
AC	357	32.6	438	37.6		**0.80 (0.67-0.95)**
CC	61	5.6	62	5.3		0.95 (0.66-1.38)
AC/CC	418	38.1	500	42.9	**0.021**	**0.82 (0.69-0.97)**
C allele	0.219		0.241			
HWE					0.355	
P trend					0.072	
ERCC5 rs17655						
GG	291	26.6	278	24.1	0.209	1
CG	556	50.8	586	50.7		0.91 (0.74-1.11)
CC	247	22.6	292	25.3		0.82 (0.65-1.04)
C allele	0.480		0.506			
HWE					0.634	
P trend					0.077	
ERCC1 rs3212986						
GG	506	46.5	528	45.9	0.918	1
TG	478	43.9	515	44.8		0.96 (0.81-1.15)
TT	104	9.6	107	9.3		1.01 (0.75-1.36)
T allele	0.315		0.317			
HWE					0.245	
P trend					0.902	

Note: P-value < 0.05 was considered statistically significant (in bold).
Pearson's chi-square test for difference in distributions between the case and control groups.
The mismatch between the number of genotyping samples and a total of samples is due to the absence of samples.
[a]Adjusted for age, gender in the logistic regression model.

TABLE 3 Stratified analysis of the SNP rs6498486 genotypes associated with colorectal cancer risk

Variables	Genotypes (cases/controls)		*P*	Adjusted OR (95% CI)[a]
	AA	AC/CC		
Gender				
Male	432/381	253/298	**0.009**	**0.75 (0.60-0.93)**
Female	246/284	164/ 202	0.635	0.94 (0.72-1.23)
Location				
Colon	331/ 666	206/ 500	0.079	0.83 (0.67-1.02)
Rectum	347/666	212/500	0.050	0.81 (0.66-1.00)
Grade				
Low	214/666	127/500	0.063	0.81 (0.64-1.03)
Intermediate/High	443/666	275/500	**0.050**	0.83 (0.69-1.00)
Depth of invasion				
T1	30/666	19/500	0.569	0.93 (0.71-1.21)
T2	120/666	66/500	0.058	0.92 (0.72-1.17)
T3	54/666	37/500	0.680	0.82 (0.64-1.04)
T4	456/666	288/500	0.071	0.74 (0.59-0.92)
Lymph node metastasis				
N0	366/666	223/500	**0.044**	**0.80 (0.66-0.99)**
N1	312/666	195/500	0.092	0.83 (0.67-1.03)
Distant metastasis				
M0	400/666	265/500	0.206	0.88 (0.73-1.07)
M1	278/666	153/500	**0.008**	**0.73 (0.58-0.91)**
TNM				
I	70/666	38/500	0.121	0.72 (0.48-1.09)
II	150/666	110/500	0.866	0.97 (0.74-1.27)
III	180/666	117/500	0.277	0.87 (0.67-1.12)
IV	278/666	153/500	**0.008**	**0.73 (0.58-0.91)**

Note: P-value < 0.05 was considered statistically significant (in bold).
Pearson's chi-square test for difference in distributions between the case and control groups.
[a]Adjusted for age, gender in the logistic regression model.

To further explore whether rs6498486 polymorphism can affect the transcriptional activity of ERCC4, we performed luciferase assay to evaluate rs6498486 transcriptional activity. We constructed two luciferase reporter vectors (Figure 1E) contain rs6498486 allele (A or C), which were transient transfected into RKO, HT-29, and 293T cells together with the Renilla luciferase plasmid. As shown in Figure 1F, the plasmid carrying C allele exhibited the significantly reduced luciferase activity than that with A allele in 293T, RKO, and HT-29 cells.

Inspired by luciferase assay, we wondered whether rs6498486 polymorphism could influence the expression level of ERCC4 through affecting the ERCC4 transcription activity. Then, we collected paraffin sections of colorectal cancer (n = 50) for each genotype and performed IHC analysis. The IHC staining of ERCC4 in colorectal cancer tissue with different rs6498486 genotypes was shown in Figure 1A,B. Results showed that the ERCC4 protein levels were downregulated in the patients carrying the AC/CC genotypes compared with those with the AA genotype. Then, we examined ERCC4 mRNA level in 88 tumor tissues from colorectal cancer patients with different genotypes. Similar to the IHC assay, compared with AA genotype, patients carrying with CC genotype showed a lower ERCC4 mRNA level (Figure 1C).

3.5 | Expression level of ERCC4 in CRC

To further investigate the potential role of ERCC4 in CRC development, we then assessed the ERCC4 expression in 88 colorectal cancer tissue and adjacent normal tissue. As shown in Figure 2A, compared with adjacent normal tissue, a higher ERCC4 mRNA level was observed in cancer tissue. In conclusion, ERCC4 rs6498486 A > C polymorphism reduced CRC risk by downregulating ERCC4 expression.

4 | DISCUSSION

In this work, we investigated the association between 3 SNPs (rs6498486, rs3212986, and rs17655) and colorectal cancer

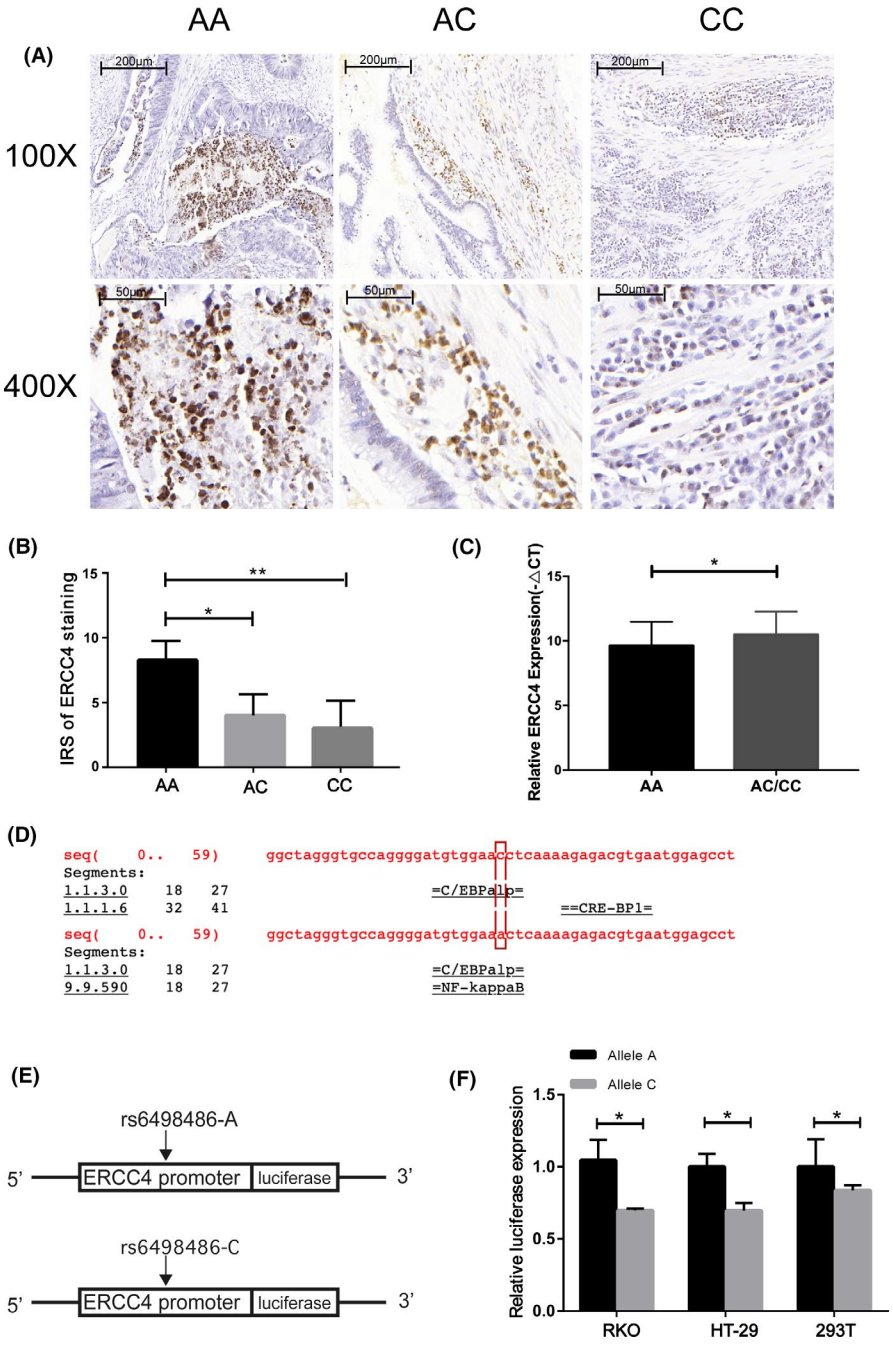

FIGURE 1 Expression of ERCC4 in colorectal cancer tissues obtained by immunohistochemical staining. Representative IHC images were obtained at 100X and 400X magnification. (A) And IHC staining scores (B) are shown. *P < 0.05, compared with AA genotype. (C) Correlation between different rs6498486 genotypes and ERCC4 expression in colorectal cancer tissue. (D) Prediction of the binding of transcription factors to the mutation region of rs6498486 with the bioinformatics algorithm (AliBaba2). (E) Schematic description of the reporter plasmids of rs6498486. (F) Reporter plasmids with different allele of rs6498486 were transfected into RKO, HT-29, and 293T cells. Then, relative luciferase activity was detected and normalized by the internal control of renilla luciferase. The data were from three independent experiments

susceptibility. By conducting a case-control study, we found that rs6498486 polymorphisms are associated with risk of colorectal cancer. The functional assay revealed that rs6498486 A > C inhibited the transcriptional activity of ERCC4, downregulated ERCC4 expressions, and ultimately decreased the risk of colorectal cancer (Figure 2B).

Many efforts have been made to investigate the role of ERCC1, ERCC4, and ERCC5 polymorphisms in the development of cancer. For example, a meta-analysis demonstrated that *ERCC1* rs3212986 polymorphism was significantly associated with glioma risk under the following genetic models, and no association was observed between glioma risk and ERCC5 rs17655.[23] However, Hou et al found that rs6498486 polymorphism not associated with risk of colorectal cancer and rs3212986 may be associated with colorectal cancer risk in a Chinese population.[20] In addition, in recently published meta-analyses, Zeng et al[24] suggests that the ERCC5 rs17655 polymorphism might contribute to genetic susceptibility to colorectal cancer. These results were inconsistent with our studies. This difference may be related to the small sample size and different ethnic backgrounds of previous researches.

Nucleotide excision repair (NER) plays a pivotal role in maintaining the stability and integrity of the genome.[25] And NER process includes steps of damage recognition, damage demarcation and unwinding, damage incision, and new strand ligation.[26,27] In the NER process, ERCC4 as an important gene could play an indispensable role in varied DNA repair system.[11] XPF (ERCC4) is located on

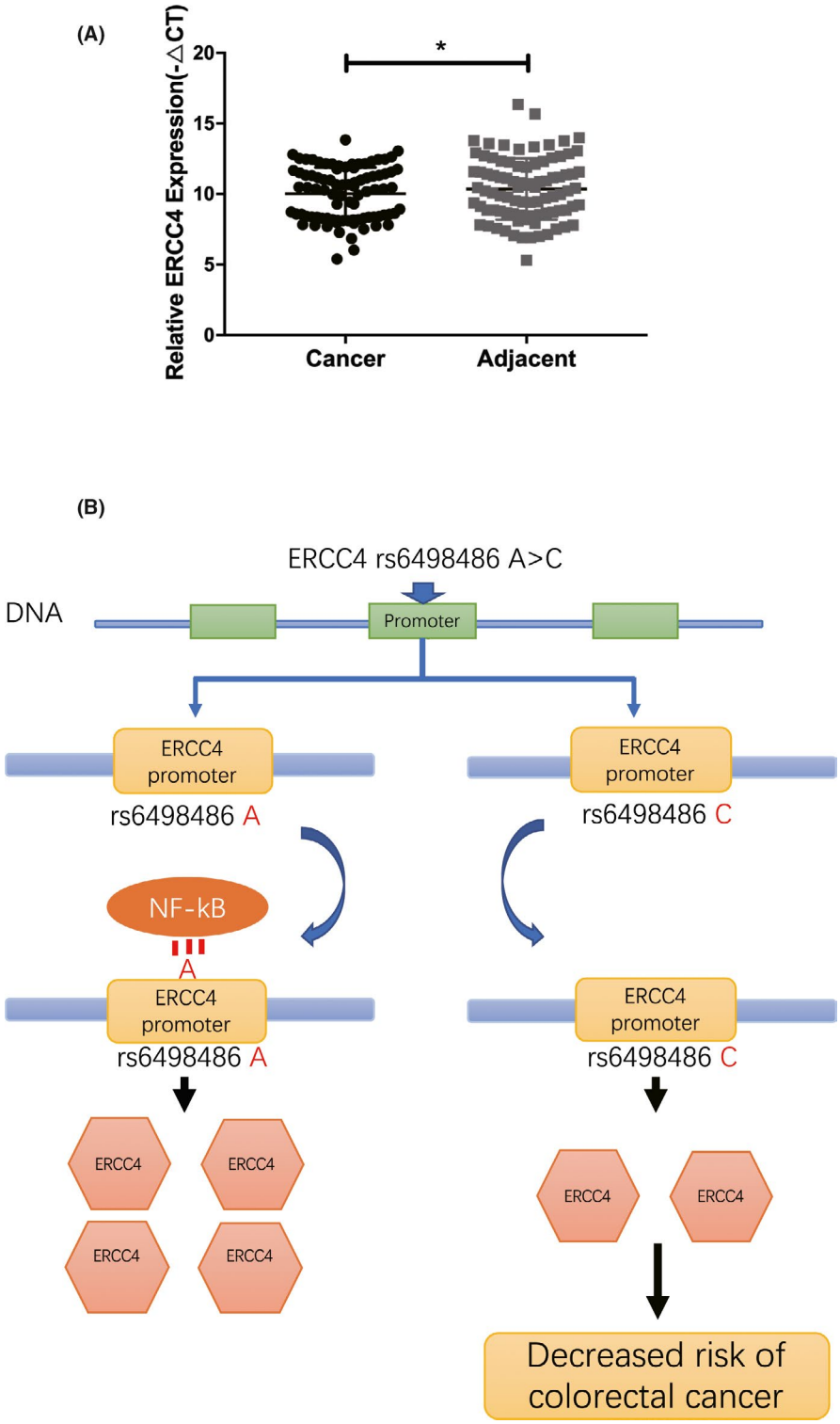

FIGURE 2 (A) Relative ERCC4 expression in colorectal cancer tissue and adjacent normal tissue. (B) Model of regulations among NF-kB, rs6498486, and ERCC4 involved in colorectal cancer development. Expression of ERCC4 depends on the binding of NF-kB, and the rs6498486 polymorphism may influence the binding ability of the transcription factor NF-kB to the rs6498486 mutation region and thus affect the transcription activity of the ERCC4 gene and decreased ERCC4 gene expression

chromosome 16p13.12, contains 11 exons and spans at approximately 28.2 kb, and is involved in the 5′ incision made in the NER pathway.[28] The ERCC1-XPF complex is two subunit structure-specific nuclease. This nuclease can cleave DNA specifically near junctions between single-stranded and double-stranded DNA, where the single strand departs 5′ to 3′ from the junction. In addition, ERCC1-XPF is a process that removes DNA lesion caused by ultraviolet (UV) radiation exposure and by DNA damaging agents that cause covalent helix-distorting adducts.[29] Therefore, ERCC4 is a core protein of NER pathway, and its expression affects DNA repair capacity. Nevertheless, in this study, we found a higher level of ERCC4 expression was observed in colorectal cancer tissue. We speculated that a large amount of DNA damage was accumulated in the tumor, and cells need expressed more ERCC4 proteins to repair these damages, which led to the protein level of ERCC4 increased in the colorectal cancer tissues. This conjecture explained that how

rs6498486 A > C polymorphism involved in colorectal cancer risk by decreased ERCC4 expression.

The nuclear factor-kappaB (NF-kB) is a family of regulatory proteins controlling many biological processes, including: proliferation, survival, apoptosis, invasion, and metastasis in many normal and cancerous cell types.[30-32] When the NF-kb pathway is activated, the released p65/p50 NF-kB dimers translocate from the cytoplasm to the nucleus where they bind to specific DNA sequences and promote transcription of target genes.[32,33] The NF-kB signaling pathway has been shown to promote the progression of different cancers, including breast cancer, prostate cancer, pancreatic cancer, and colorectal cancer.[34,35] In our study, we found that rs6498486 A > C polymorphism may alter the binding of transcription factors (NF-kB) to promoter regions. Similarly, luciferase assay demonstrated that rs6498486 polymorphism affects the transcription activity of the ERCC4 gene. However, further functional experiments are required to demonstrate the ERCC4 rs6498486 polymorphism might change the binding affinity of the transcription factor NF-kb to the rs6498486 mutation region.

In conclusion, our results revealed that rs6498486 A > C polymorphism could alter the transcription activity of ERCC4 gene, thereby changing the expression level of ERCC4. However, further functional researches are required to verify our result.

AUTHOR CONTRIBUTIONS

All authors contributed significantly to this work. Yujie Zhang and Shenshen Wu conceived and designed the study; Xiumei Zhou, Fang Huang, Rui Chen, Jiong Wu, and Yigang Wang analyzed the data; Yujie Zhang, Shenshen Wu, Jiong Wu, and Yigang Wang created all tables and figures; Yujie Zhang drafted the manuscript; Shenshen Wu, Jiong Wu, and Yigang Wang made critical revision of the manuscript. All authors read and approved the final manuscript. Xiumei Zhou, Fang Huang, and Rui Chen monitored the study.

INFORMED CONSENT

In this study, all activities on human subjects were carried out under full compliance with the Helsinki Declaration, and all individual participants signed an informed consent form.

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