



## Investigation of *cullin-3* gene expression changes in people with colorectal cancer and polyps in China

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### ABSTRACT

Colorectal cancer (CRC) is one of the most common fatal malignancies caused by environmental and genetic factors. Considering the increasing frequency of CRC worldwide, especially in China, the importance of research on CRC is more widely defined. A recent study focused on molecular pathways involved in colon cancer carcinogenesis to improve cancer diagnosis and treatment to identify new biomarkers. Colon cancer is the result of dysplasia in primary growths of the intestine, known as polyps. These early growths are unknown and different in terms of morphology, molecular mechanisms, and the ability to cause colon cancer. This study aims to investigate the expression level of the *CUL3* gene in polyps and colorectal cancer. This cross-sectional study collected 300 colorectal tissue biopsy samples, including 40 tumor tissue samples, 73 precancerous lesions with their adjacent tissue, and 31 normal tissue samples. The expression of the *CUL3* gene was investigated by the Real-time PCR method. There was no significant difference in *CUL3* mRNA expression between polyp tissues and their adjacent samples ( $p = 0.41$ ). Our results showed no statistically significant difference in *CUL3* gene expression between tumor tissues and their adjacent thermal samples ( $p = 0.78$ ) and between tumor and polyp groups ( $p = 0.53$ ). *CUL3* may play an essential role in regulating cancer and CRC progression by stimulating the proteasomal degradation of various tumor suppressors or oncogenes. Studies on the effective substrates of *CUL3* in colorectal cancer are essential.

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### Introduction

Cancer is a genetic disorder in which the normal control of cell growth and division is lost, and it includes all types of malignant tumors, known as neoplasms. Colorectal Cancer (CRC), also known as colon or rectal cancer, is the most common malignant tumor that develops from epithelial cells in the colon or rectum (1). CRC is the third most common type of cancer worldwide. The incidence rate of colon and intestinal cancer by the National Cancer Institute of America is estimated to be 236,830 cases by the end of 2024 (2). The death rate due to it is about 100,000 people. It is estimated that there will be over 1.9 million newly diagnosed CRC patients and nearly one million CRC-related deaths in 2020 globally, accounting for approximately one-tenth of cancer cases and deaths. The burden of CRC is also crucial in China, ranking as the fourth leading cause of cancer-related deaths (1).

The risk of CRC in the general population is about 5%, which gradually increases with age (2). Based on the development of genome-based technologies, it has been determined that CRC can be considered a heterogeneous disease with different survival rates, including a 90% five-year survival rate for patients in the early stages of cancer and 10% for those with metastatic cancer (3). It indicates the urgent need to identify useful biomarkers for early diagnosis of this disease (4). Benign masses are called colon polyps. Colon polyps are overgrowths of squamous cells of the intestinal mucosa, which occur in the wall of

the colon or rectum (5). Neoplastic polyps can end up in CRC; the event that causes this transformation is related to the sequence of adenoma to carcinoma. Colon cancer is caused by the accumulation of genetic and epigenetic changes in the epithelial cells of the colon, which turns them into carcinoma (6).

NRF2-KEAP1 is one of the most important pathways for cell survival and defense. NRF2 protects cells and tissues from various toxic drugs and carcinogens by increasing the expression of several cytoprotective genes (7). *CUL3* is a substrate receptor for KEAP1, which targets the transcription factor NRF2 for ubiquitination and degradation. As a result, the destruction of NRF2 by *CUL3* is very significant in response to cellular stress that controls the survival of normal and cancer cells (8). Cullin-RING multiprotein complexes constitute the most prominent family of ubiquitin ligases in which a specific Cullin is used as a scaffold to link two functional units (9). The human Cullin family comprises eight members, including *CULAA*, *CUL3*, *CUL2*, *CUL1*, *CULT*, *CUL*, *CUL4B*, and *CUL9*. As a *CUL* adapter, the BTB domain-containing protein acts to bind to the substrate (10). *CUL3* is highly functional for multiple protein ligase-ubiquitin complexes involved in regulating the target protein's ubiquitination and proteasomal degradation activities. Colon cancer patients face a lack of clinical symptoms until the end stages, leading to late diagnosis, poor prognosis, and increased mortality (11). Early diagnosis in patients leads to treatment in 80% of cases, so identifying suitable tools for colon cancer

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screening that are simple, affordable, specific, and sensitive is a primary priority (9).

Due to the potential role of the NRF2/KEAP1 pathway in tumorigenesis, growth survival of metastatic cancer cells, as well as its role in drug resistance, the expression of the critical gene *CUL3* involved in this pathway was examined in all types of colon polyps and tumor tissues and healthy tissues and comparing this information with clinicopathological characteristics (8). And compiling information on the role of the activity of these genes in the development of colorectal cancer from the polyp stage to the development of metastatic cancers was targeted. Also, examining the tissue specificity of the changes and the presentation of biomarkers for prognosis and diagnosis in early precancerous growths of the colon and in colorectal cancer were considered.

## Materials and Methods

### Study population and sampling process

This cross-sectional study collected and studied 300 pathology samples (40 tumor samples, 73 polyp samples along with their adjacent tissues, and 31 normal tissue samples) related to patients with digestive symptoms. The criteria for entering the study were colonoscopy for both groups, a positive pathology result for the patient group, and a negative pathology result for the healthy group. None of the patients had undergone chemotherapy or radiotherapy and did not have other types of cancer. Also, patients who did not have Chinese citizenship were excluded from the study. The samples included fresh tissue from intestinal polyps, including primary polyps, and new tissue from a cancerous intestinal tumor, and normal tissue was removed from a distance of 1 to 10 cm. All samples were examined histopathological, and the pathologist reported related changes. According to the pathologist group, patients' tissue samples were divided into three samples, typical polyp, and tumor. Clinical and demographic information, including age, sex, diabetes, smoking, blood pressure, family history, inflammatory bowel disease (IBD), type of neoplasm, and pathology of the place of occurrence after the colonoscopy process, were obtained from the patients' documents.

### Extracting RNA from fresh tissue and determining the quality and concentration of extracted RNA by photometric method

RNA extraction from tissue samples was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the relevant protocol. The purity of the extracted RNA samples and the optimal and appropriate concentration of RNA were checked. In this regard, Thermo 2000 Scientific NANODRAP device was used to determine the concentration of RNA samples. Samples with A260/A280

ratio between 1.8 and 2 were used to make cDNA.

### The cDNA synthesis by RT-PCR technique

Two micrograms of total RNA were extracted from each sample using random hexanucleotide primers with the help of Thermo Scientific RevertAid Reverse Transcriptase kit and stored at  $-70^{\circ}\text{C}$ . In the next stage of cDNA production, the primer design was done.

### Primer design

The desired gene sequences were obtained from the NCBI database, and the primers were designed using Primer Blast software. In this study, it was tried to design upstream and downstream primers on separate exons or exon boundaries to prevent genomic DNA replication.

### Real-time PCR

A quantitative real-time polymerase chain reaction (qRT-PCR) technique was used to check the expression levels of the *CUL3* gene, which was performed using a commercial kit SYBER Premix Ex Taq II (Takara) and Real-Time 6000 CR Rotor gene device. The relative expression level of the genes was evaluated by comparing the *BACTIN* reference gene as an internal control (Table 1).

Real-time PCR temperature cycle conditions for the *CUL* gene were as follows:

A step of  $95^{\circ}\text{C}$  for 30 seconds for the initial activation of the polymerase enzyme, followed by 40 cycles that included  $95^{\circ}\text{C}$  for 5 seconds and  $58^{\circ}\text{C}$  for 30 seconds, the annealing step. And for the extension stage, 72 degrees Celsius was defined for 25 seconds. Finally, the melting curve was analyzed from 72 to  $97^{\circ}\text{C}$  with an increase in the rising value of  $0.5^{\circ}\text{C}$  in each cycle.

### Statistical analysis

Statistical analysis was analyzed using SPSS version 19 statistical software. Quantitative information was shown in the form of mean  $\pm$  standard deviation. To analyze the results related to quantitative expression, gene expression information of the *CUL3* gene was normalized in comparison with  $\beta$ -*ACTIN* as a reference gene.

Analytical information was displayed using the  $2^{-\Delta\Delta\text{Ct}}$  method and its log. Graph Pad Prism7 (Graph Pad Inc., USA) was used for statistical gene expression analysis. The significance of the gene expression differences between the control and case groups was estimated by a statistical T-test.  $P < 0.05$  was considered statistically significant.

## Results

### General statistical information of the study subjects

This study was conducted on 300 samples, including colorectal and adjacent tissues (64 men and 60 women).

**Table1.** Primer sequences, product length, and annealing temperature for the *CUL3* gene and  $\beta$ -*ACTIN* as a reference gene.

Gene	Primer Sequence (5'-3')	Product length	Annealing temp.
<i>CUL3</i>	F: ACCCAAGGTTCTTACCAGCG	86 bp	$58^{\circ}\text{C}$
	R: TCACCTGTCTCTGGCGGAT		
$\beta$ - <i>ACTIN</i>	F: CCTATGATTCGTGGGCGACGA	115 bp	$60^{\circ}\text{C}$
	R: GTGGGGTGTTCATGGCGA		

**Table 2.** Frequency of clinicopathological and demographic characteristics of Chinese patients with and without CRC.

Variable	Polyp (n=73)	Tumor (n=40)	Normal (n=31)	P-value
<b>Age (year)</b>	60.4 ± 11.3	59.1 ± 13.2	58.8 ± 10.1	0.66
<b>Body Mass Index</b>	26.0 ± 1.2	24.8 ± 3.1	25.5 ± 2.6	0.31
<b>Gender</b>				
Female	42 (57.53%)	29 (72.5%)	21 (67.74%)	0.17
Male	31 (42.47%)	11 (27.5%)	10 (32.26%)	
<b>Disease Family History</b>				
No	59 (80.82%)	33 (82.5%)	28 (90.32%)	0.63
Yes	14 (19.18%)	7 (7.5%)	3 (9.68%)	
<b>Smoking</b>				
No	66 (90.41%)	27 (67.5%)	25 (80.68%)	0.19
Yes	7 (9.59%)	13 (32.5%)	6 (9.32%)	
<b>Position</b>				
Colon	68 (93.15%)	22 (55%)	22 (70.96%)	0.009
Rectum	5 (6.85%)	18 (45%)	9 (29.04%)	
<b>Blood Pressure</b>				
No	67 (91.78%)	33 (82.5%)	29 (96.66%)	0.53
Yes	6 (8.22%)	7 (7.5%)	2 (3.34%)	
<b>Inflammatory intestinal diseases</b>				
No	62	35	30	0.17
Yes	11	5	1	
<b>Diabetes</b>				
No	69 (94.52%)	39 (97.5%)	29 (93.54%)	0.22
Yes	4 (5.48%)	1 (2.5%)	2 (6.46%)	

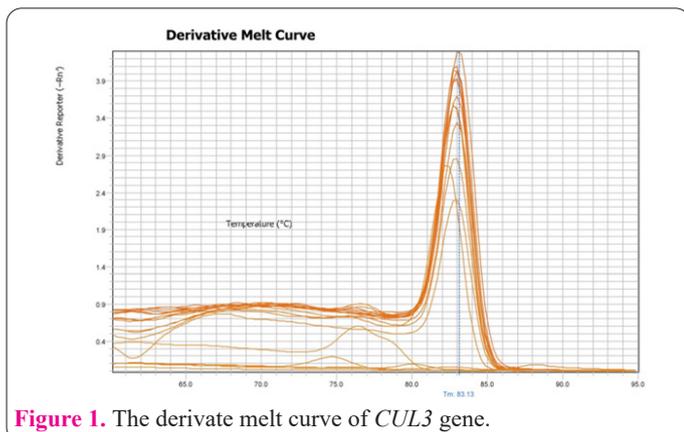
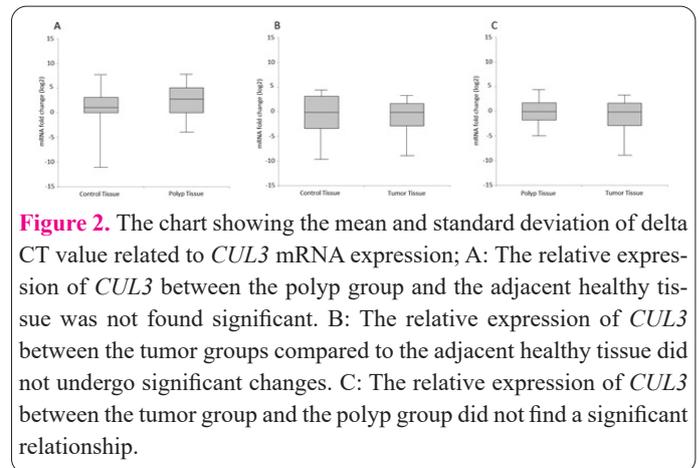
The average age of patients with polyps and tumors and normal subjects was  $6.58 \pm 7.12$ ,  $61.7 \pm 13$ , and  $3.57 \pm 2.9$  years, respectively. Also, the body mass index (BMI) in the above groups was  $2.25 \pm 8.2$ ,  $9.2 \pm 9.2$ , and  $3.26 \pm 8.3$  kg/m<sup>2</sup>, respectively. More details about demographic and clinic-pathological characteristics are given in Table 2. As shown in Table 2, the samples included 84 samples (7.73%) from the colon and 30 samples (3.26%) from the rectum. Most of the participants in this research did not have a family history of diabetes, blood pressure, and inflammatory bowel diseases.

### RT-PCR

The proliferation and melting curve related to the *CUL3* gene can be seen in Figure 1, which shows the specificity of the real-time PCR conditions for this gene.

### *CUL3* gene expression pattern in the tumor, polyp, and normal tissue samples

The comparison of the *CUL* gene expression changes

**Figure 1.** The derivate melt curve of *CUL3* gene.**Figure 2.** The chart showing the mean and standard deviation of delta CT value related to *CUL3* mRNA expression; A: The relative expression of *CUL3* between the polyp group and the adjacent healthy tissue was not found significant. B: The relative expression of *CUL3* between the tumor groups compared to the adjacent healthy tissue did not undergo significant changes. C: The relative expression of *CUL3* between the tumor group and the polyp group did not find a significant relationship.

in the polyp group compared to the adjacent normal group, which is normalized against BACTIN as a reference gene, is shown in Figure 2-A. No significant difference was observed in the *CUL3* mRNA expression of polyp tissues and their adjacent samples ( $p = 0.41$ ). In the comparison of *CUL3* gene expression in adjacent tumor and normal samples, *CUL3* mRNA expression in the tumor group had no significant changes compared to normal ( $p = 0.78$ ) (Figure 2-B). In comparing *CUL3* mRNA expression in tumor and polyp samples, the difference in *CUL3* mRNA expression was not statistically significant ( $p = 0.53$ ) (Figure 2-C).

### Discussion

Despite many advances in various sciences in recent years, cancer is still considered one of the most critical threats to human life (12). Colorectal cancer (CRC) is the

third most common malignancy leading to cancer-related death worldwide, so it ranks first among digestive tract cancers even before stomach cancer (13). According to an estimate made in 2018, colorectal cancer was the cause of 1.2 million new cancer cases and the cause of 608,700 deaths worldwide this year (14). The prevalence of colon cancer is also increasing in China, and due to the high proportion of young people in China, it is expected that in the coming years, due to the aging of the country's population, we will see the growth of the prevalence of various types of cancer, such as colorectal cancer (15). Although colonoscopy is currently the most accurate method for colorectal cancer screening, this method is invasive and complicated, and expensive (12, 16).

On the other hand, early detection of colon neoplasms is potentially important because it reduces mortality and increases the survival rate in CRC patients (16). Therefore, finding a biomarker to detect cancer in the early stages is a significant concern in this field. Proteolysis of cellular proteins dependent on ubiquitin plays a vital role in maintaining the balance between average growth and uncontrolled proliferation (8). CUL3 is one of the ubiquitin ligases belonging to Cullin-RING multi-protein complexes. KEAP1 is the CUL substrate adapter. Under basal conditions, the CUL3 KEAP1 complex can target NRF2 and lead to ubiquitin-dependent degradation (17). As a result, the destruction of NRF2 by CUL3 in response to cellular stress that controls the survival of normal and cancer cells is very significant and can cause profound effects on tumorigenesis. Based on the studies, it has been found that CUL is often decreased in some human malignancies, such as bladder cancer (18), breast cancer (19), prostate cancer (20), and colon cancer (21).

It has also been shown that CUL3 plays a role as a tumor suppressor gene in lung cancer (22) and colon cancer (21). Recently, Martinez *et al.* (23) reported that CUL3 and the NRF2 pathway were associated with poor prognosis in head and neck squamous cell carcinoma. Decreased expression of CUL3 can profoundly affect tumorigenesis by affecting the NRF2 pathway. However, conflicting reports have also been presented in several malignant tumors with CUL3 overexpression to tumor behavior and therapeutic responses. Grau *et al.* (18) showed that overexpression of CUL is responsible for progression, metastasis, and poor clinical outcome in patients with bladder cancer. Similarly, data collected by Huo *et al.* (24) showed that increased expression of CUL3 is associated with invasion and metastasis in breast cancer. On the other hand, Wang and his colleagues showed that CUL3 plays a vital role in colon cancer cell proliferation by targeting methionine adenosyl transferase IIa for degradation by ubiquitination (21). Another substrate of CUL3-KEAP1 ubiquitin ligase is IKK, which plays a role in tumor development and progression by activating the NF- $\kappa$ B pathway. Removing KEAP1, Lee *et al.* (15) led to the stabilization and accumulation of IKKB and the subsequent increase in NF- $\kappa$ B-dependent angiogenic factors. Therefore, CUL3-mediated disruption of IKK ubiquitination is probably an influential factor in tumorigenesis. Based on our results, the expression of CUL3 in colon neoplastic tissue did not show a significant relationship.

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