Brief Report

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C35 is overexpressed in colorectal cancer and is associated tumor invasion and metastasis

Xuan Dong[1,2], Yong Huang[1], Lingbin Kong[3], Jin Li[1], Jingxuan Kou[1], Lixin Yin[1], Jingyu Yang[1,*]

[1] *Shandong Academy of Medicine Sciences, Shandong Provincial Institute of Parasitic Diseases, Ji'ning, Shandong, China;*
[2] *School of Medicine and Life Sciences, Ji'nan University, Shandong Academy of Medicine Sciences, Ji'nan, Shandong, China;*
[3] *Ji'ning Medical University, Ji'ning, Shandong, China.*

Summary **The aim of this study was to investigate the expression of *C35*, an oncogene previously found in breast and prostate cancers, and its clinicopathological significance in colorectal cancer (CRC). Qualitative and quantitative detection of C35 mRNA expression was performed using reverse transcription-PCR (RT-PCR) and real-time PCR. C35 protein expression was determined using immunohistochemistry. C35 mRNA was detected in none of 10 normal colorectal tissue samples, 55 of 65 (84.6%) CRC tissue samples, and 43 of 55 (78.2%) adjacent non-cancerous tissue samples. In addition, the level of C35 mRNA in CRC tissue samples was markedly higher than that in tumor adjacent non-cancerous tissue samples. C35 protein expression was detected in 58 of 80 (72.5%) CRC tissue samples and was closely associated with tumor serosal invasion, lymphnode metastasis, and an advanced Dukes stage. These results suggest that C35 might serve as a biomarker or therapeutic target for management of CRC.**

Keywords: Therapeutic target, biomarker, invasion and metastasis, cancer

1. Introduction

According to statistics from the World Health Organization published in 2012, colorectal cancer (CRC) was the third most common form of cancer in men (746,000 patients, 10.0% of all patients with cancer) and the second in women (614,000 patients, 9.2% of all patients with cancer) worldwide (*1*). In China, the incidence of CRC has been increasing in recent years as living conditions improve and eating habits change, with both sexes accounting for an estimated 253,000 patients in 2012 (*1*). Early detection and treatment of CRC is critical and is significantly associated with patient prognosis (*2*). Cancers that are confined within the wall of the colon may be cured with surgery while a tumor that has already spread is usually not curable and chemotherapy instead focuses on improving the patient's quality of life and symptoms (*3-5*). Individualized treatment with targeted agents such as cetuximab has many advantages over traditional cytotoxic agents and represents the future of CRC treatment (*6*). Accordingly, identification and characterization of cancer-specific biomarkers has enormous potential to facilitate detection and treatment of CRC.

The *C35* gene, located on human chromosome 17q12, encodes a 12 kDa membrane-anchored protein that functions as an oncogene in several types of cancer (*7-10*). Evans *et al*. found that C35 was highly expressed in breast carcinoma in comparison to adjacent normal breast epithelium (*11*). The same study also examined its expression in 38 normal human tissue samples, including blood, bone marrow, and gastrointestinal tissue, and it found that none of the normal tissue samples were positive for C35 expression, with the exception of Leydig cells in the testes (*11*). Similarly, a study by Vishwanatha *et al*. found that C35 was highly expressed in prostate cancer cell lines and tumors but minimally expressed in normal prostate cells and tissues (*8*). The difference in expression in cancerous tissue and normal tissue suggests that C35 may play a role in transforming healthy cells in cancer initiation and progression and thus has potential value in cancer management (*11*). The expression profile of C35 in CRC is not yet known. The present study examined C35 expression in CRC and investigated its clinicopathological significance.

**Address correspondence to:*
Dr. Yingyu Yang, Shandong Provincial Institute of Parasitic Diseases, Ji'ning 272033, Shandong, China.
E-mail: yangjingyu3368@sina.com

2. Materials and Methods

2.1. *Materials*

Cancer tissue samples and tumor-adjacent tissue samples were obtained via open surgery or endoscopic surgery from the Third Affiliated Hospital of Shandong Academy of Medical Sciences with the informed consent of patients. These samples were subjected to reverse transcription-PCR (RT-PCR) and real-time PCR. Cancer tissue samples and normal colorectal tissue samples were also obtained from King Med Diagnostics (Ji'nan, Shandong, China). These samples were subjected to immunohistochemistry. The study protocol was approved by the Hospital Ethics Review Committee. None of the patients received preoperative treatment.

2.2. *RT-PCR*

Total RNA was isolated using an RNAprep pure Tissue Kit (Tiangen Biotechco., Beijing, China). The OD260/OD280 ratio of total RNA extracted from tissue samples ranged from 1.8 to 2.0. First-strand cDNA synthesis was done using TIANScript RT Kit (Tiangen Biotechco.). Primer sequences were as follows: for GAPDH (glyceraldehyde-3-phosphate dehydrogenase), sense, 5'-GACTCACCCTGCCCTCAATA-3'; antisense, 5'-CCCTGTAGCCTGGACCTGAT-3'; for C35, sense, 5'-GCCATCCGAAGAGCCAGTA-3'; antisense, 5'-ATTACCGAGGCGAAGAGTGG-3'. PCR cycling parameters were: at 95°C for 3 min for pre-denaturation, followed by 40 cycles at 94°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec, and a final cycle at 72°C for 5 min for extension. GAPDH was used as an internal control. PCR products (10 μL) were analyzed electrophoretically using 2% agarose gel electrophoresis and viewed under ultraviolet (UV) light. The remaining products were sent to Shanghai Sangon Biotech for sequencing.

2.3. *Real-time PCR*

Real-time PCR was performed on all of the cDNA synthesized from samples with *C35* gene expression. Primers were the same as used in RT-PCR. Real-time PCR was performed using a SYBR Green Kit (Takara Biotechco., Dalian, Liaoning, China). The reaction system included 10 μL of SYBR® Premix Ex Taq (2×) (Tli RnaseH Plus), 0.4 μL of PCR forward primer, 0.4 μL of PCR reverse primer, 0.4 μL of ROX Reference Dye (50×) II, 2 μL of sample, and 6.8 μL of dH2O. PCR cycling parameters were: at 95°C for 3 min for pre-denaturation, followed by 40 cycles at 95°C for 5 sec and 60°C for 34 sec. Amplification was performed with an ABI 7500 Sequence Detection System. All reactions were performed in triplicate, and GAPDH served as an internal control. The results were quantified as Ct values, where Ct is defined as the threshold cycle of PCR at which the amplified product is first detected and the values are expressed as the ratio of the target to the control.

2.4. *Immunohistochemistry*

In total, 80 paraffin-embedded colorectal carcinoma (grade 1, 2, and 3) and 10 normal tissue samples were received from King Med Diagnostics. None of the patients were undergoing chemotherapy or radiotherapy before radical surgery. Patient age ranged from 46 to 87 years, with a median of 67 years and a mean age of 69 years. Sections were viewed using light microscopy and staging was confirmed independently by two qualified pathologists. Mouse anti-XTP4 monoclonal antibody (Abcam, Cambridge, MA, USA) and a GTvisionTMIII anti-mouse universal immunohistochemical detection kit (Gene Tech Company, Shanghai) were used in this study. Immunohistochemistry studies were performed using a Maxvision two-step method. Consecutive 3-μm sections from paraffin-embedded tissue were deparaffinized, hydrated, and rinsed in distilled H_2O. The sections were then boiled in citrate buffer (pH 6.0) in a high pressure cooker for 5 min and cooled to room temperature. Afterwards, they were washed 4 times with phosphate-buffered saline (PBS; pH 7.4) for 3 min. All of the sections were then incubated with the primary mouse anti-human monoclonal anti-XTP4 antibody (1:100 diluted) overnight at 4°C. The next day, the sections were washed 4 times in PBS for 3 min, and they were then incubated with anti-mouse IgG antibody for 25 min. Afterwards, the sections were washed 4 times with PBS for 3 min. DAB chromogenic reagent was dropped on sections and staining was observed under a light microscope. After staining, the sections were rinsed in distilled water to stop the reaction. After counterstaining with hematoxylin, the sections were dehydrated and mounted. PBS instead of the primary antibody was used as a negative control.

A staining index was devised by multiplying the intensity of positive staining by the proportion of stained cells. The staining intensity was graded using the following standard: 0 for unstained cells, 1 for cells stained yellow, 2 for cells stained orange, and 3 for cells stained brown. The proportion of stained cells was based on the following criteria: 0 for ≤ 5% staining, 1 for 6-25%, 2 for 26-50%, 3 for 51-75%, and 4 for > 75% staining. The staining scores of 0-1 point were considered negative, scores of 2-4 points were considered weakly positive, scores of 5-8 points were considered moderately positive, and scores of 9-12 points were considered strongly positive.

2.5. *Statistical analyses*

Statistical analysis was performed using the Statistical

Package for the Social Sciences (SPSS), version 17.0 (USA). Statistical analysis of RT-PCR results was performed using a Mann-Whitney U test. A possible association between positive expression of C35 and clinicopathological parameters was compared using a χ^2 test or Fisher's exact test. Differences with a p less than 0.05 were considered to be statistically significant.

3. Results and Discussion

3.1. *Qualitative detection of C35 mRNA expression*

RT-PCR was used to examine C35 mRNA expression in normal colorectal tissue samples (n = 10), colorectal carcinoma tissue samples (n = 65), and tumor-adjacent tissue samples (n = 55). Results indicated that 55 of 65 (84.6%) colorectal carcinoma tissue samples and 43 of 55 (78.2%) tumor-adjacent tissue samples were positive for C35 expression (Figure 1). In contrast, none of the normal colorectal tissue samples were positive for C35 expression (Figure 1).

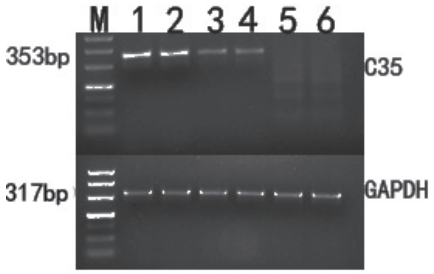

Figure 1. RT-PCR analysis of C35 mRNA expression. M, DNA marker; **1** and **2**, colorectal cancer tissue; **3** and **4**, paired adjacent-tumor tissue; **5** and **6**, normal colorectal tissue. GAPDH was used as internal control for parallel analysis of the same sample. Products from amplification of target mRNA (about 353 bp) and GAPDH mRNA (about 317 bp) were analyzed electrophoretically using 2% agarose gel electrophoresis followed by gel extraction and sequencing. Products from amplification of target mRNA were consistent with the C35 gene sequence.

3.2. *Quantitative detection of C35 mRNA expression*

Real-time PCR was performed to further explore whether *C35* gene expression differs in cancer tissue and adjacent tissue. Specimens that contained both cancer tissue and adjacent tissue (n = 50) were selected, and mRNA expression was determined and compared in cancerous and adjacent non-cancerous tissues. Results indicated that the level of C35 mRNA in cancer tissue was 5- to 10-fold higher than that in adjacent tissue. The level of C35 mRNA differed significantly in cancerous and adjacent non-cancerous tissues (p < 0.05).

3.3. *C35 protein expression and its clinicopathological significance*

Immunohistochemistry was used to examine C35 protein expression in 80 CRC tissue samples and 10 normal colorectal tissue samples. Results indicated that there was no C35 expression in normal colorectal tissue. In contrast, 58 of 80 CRC tissue samples (72.5%) displayed varying levels of C35 expression (Figure 2). C35 protein was mainly expressed in the cytoplasm, appearing as brown granules (Figure 2). The association between C35 expression and clinicopathological parameters was analyzed. C35 expression was more prevalent in CRC with serosal

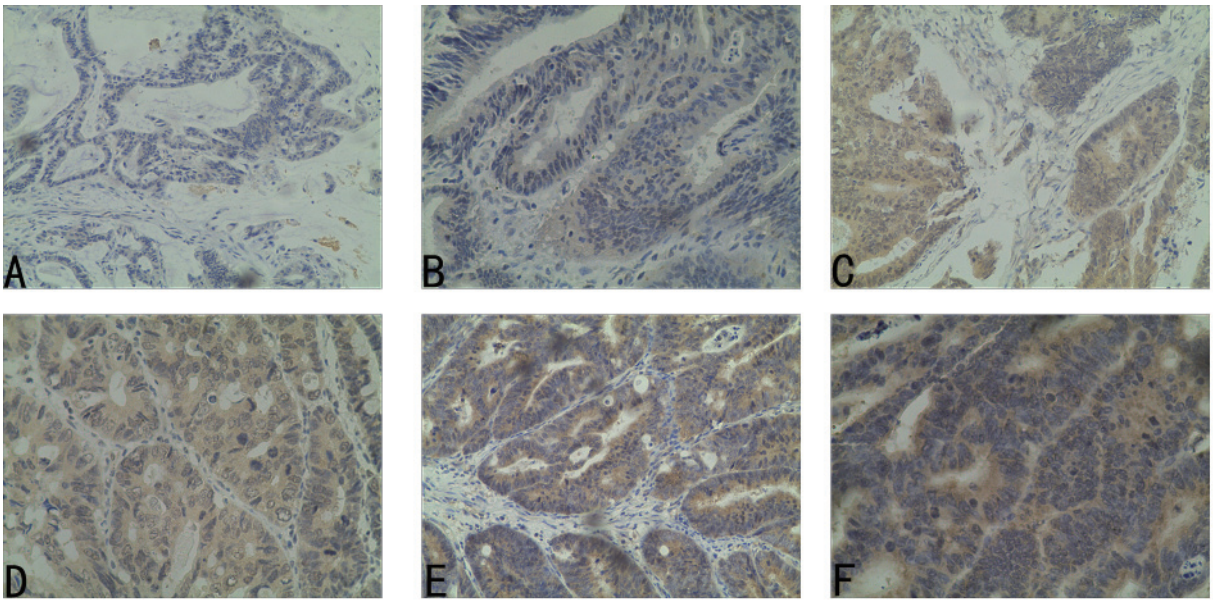

Figure 2. Expression of positivity for the C35 protein as determined by immunohistochemical staining of CRC tissue. A and **B**, weakly positive expression in CRC tissue samples (SP ×100, SP ×200); **C** and **D**, moderately positive expression in CRC tissue samples (SP ×100, SP ×200); **E** and **F**, strongly positive expression in CRC tissue samples (SP ×100, SP ×200).

Table 1. Association between C35 expression and clinicopathological parameters for colorectal cancer

Clinicopathological parameters	C35			Positive rate (%)	χ^2	*p* value
	n	+	-			
Gender						
Male	44	32	12	79.5	0.003	0.960
Female	36	26	10	72.2		
Age (years)						
< 60	18	16	2	88.9	2.158	0.142
≥ 60	62	42	20	67.7		
Tumor location						
Colon	42	32	10	76.2	0.604	0.437
Rectum	38	26	12	68.4		
Tumor diameter (cm)						
< 4	24	18	6	75.0	0.107	0.743
≥ 4	56	40	16	71.4		
Differentiation						
Well differentiated	49	34	15	69.1	0.615	0.433
Poorly differentiated	31	24	7	77.4		
Serosal invasion						
No	35	20	15	57.1	7.36	0.007**
Yes	45	38	7	84.4		
Lymphnode metastasis						
No	58	39	19	67.2	4.615	0.032*
Yes	22	20	2	90.9		
Dukes stage						
A+B	36	22	14	61.1	4.258	0.039*
C+D	44	36	8	81.8		

*$p < 0.05$; **$p < 0.01$

invasion (84.4%) than in CRC without serosal invasion (57.1%) (p = 0.007). In addition, CRC with lymphnode metastasis tested positive for C35 expression (90.9%) at a significantly higher rate than did CRC without lymphnode metastasis (67.2%) ($p = 0.032$). A higher level of C35 expression was noted with an advanced Dukes stage (C + D, 81.8%) in comparison to an early Dukes stage (A + B, 61.1%) ($p = 0.039$). There was no significant association between C35 expression and other clinicopathological parameters such as gender, age, tumor location, tumor diameter, and tumor differentiation ($p > 0.05$) (Table 1).

The present study investigated the expression profile of C35 at both the mRNA and protein levels in CRC tissue, adjacent non-cancerous tissue, and normal colorectal tissue. C35 mRNA was detected in none of 10 normal colorectal tissue samples, 55 of 65 (84.6%) CRC tissue samples, and 43 of 55 (78.2%) adjacent non-cancerous tissue samples. The level of C35 mRNA in CRC tissue was markedly higher than that in adjacent non-cancerous tissue. C35 protein expression was detected in 58 of 80 CRC tissue samples and was associated with tumor serosal invasion, lymphnode metastasis, and an advanced Dukes stage. These results suggest that C35 might have potential to serve as a biomarker or therapeutic target for CRC management.

The present study found that C35 expression was associated with invasion and metastasis of CRC, which suggests that C35 might play a role in this process. Previous studies indicated that overexpression of C35 in prostate cancer functionally enhanced migration and invasion of prostate cells (*8*). In contrast, downregulation of C35 by RNA interference reduced migration and invasion of prostate cells (*8*). The underlying mechanism for changes in C35 expression are a result of C35 acting as a signaling molecule to increase the invasive potential of prostate cancer cells by activating NF-κB-mediated downstream target genes, including matrix metalloproteinase 9, urokinase plasminogen activator, and vascular endothelial growth factor (*8*). These findings may explain why C35 expression is more prevalent in invasive CRC and they may provide clues for further research into the role of C35 and its underlying mechanism in the progression of CRC.

The present results indicated that C35 is predominantly expressed in CRC tissue in comparison to normal colorectal tissue. Thus, C35 may serve as a target for therapy. Sequencing of C35 revealed a 'CaaX' prenylation motif consisting of the last four amino acids, 'CVIL,' at the C-terminal end (*8*). The 'CaaX' group of proteins are known to be farnesylated by the enzyme farnesyltransferase or geranylgeranylated by the enzyme geranylgeranyl transferase type I (GGTase-I) (*12*). If 'X' is leucine, then protein is preferentially modified by the enzyme GGTase-I, suggesting C35 is prenylated preferentially by the enzyme GGTase-I (*12*). Key aspects of the prenylation of a 'CaaX'-type protein are its location at the cytoplasmic face of cellular membranes and its action to promote cell proliferation, migration, invasion, and metastasis (*13*). Therefore, interference with C35 prenylation may suppress that action and thus inhibit the progression of CRC. This indicates the potential for using GGTase-I inhibitors in CRC therapy. A point worth noting is that stains like simvastatin are capable of inhibiting the proliferation and migration of colon cancer cells (*14,15*), while geranylgeraniol, which can be taken up by cells and converted to geranylgeranyl diphosphate, may reverse the anti-tumor effects of stains (*15*). These findings suggest that the effects of stains on colon cancer cells might be mediated by interfering with geranylgeranylation of certain proteins. The effect of statins on C35 and the efficacy of these drugs on CRC should be studied in the future.

In conclusion, the present results indicated that C35 is predominantly expressed in CRC tissue in comparison to normal colorectal tissue and tumor-adjacent non-cancerous tissue. Furthermore, its expression is closely associated with invasive behavior by CRC. These results suggest the potential for utilizing C35 as a biomarker or therapeutic target for CRC management. C35 expression should be analyzed in a larger sample, and its biological action in CRC progression and drugs targeting this molecule should be studied in the future.

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