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Function of the macrophage-capping protein in colorectal carcinoma

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Abstract. To investigate the role of macrophage-capping protein (CapG) in the development and progression of colorectal carcinoma (CRC), immunohistochemistry (IHC), Kaplan-Meier survival analysis, wound healing and Transwell migration assays were performed. The IHC results demonstrated that CapG was relatively highly expressed in CRC tissue compared with non-tumor tissue (P<0.001), and that the expression of CapG was significantly associated with the tumor site, differentiation, lymph node metastasis and clinical stage (P=0.021, P=0.036, P=0.012 and P=0.009, respectively). Wound healing and Transwell migration assays demonstrated that the reduction of CapG expression in a CRC cell line by RNA interference was associated with significantly impaired motility (P<0.001). Kaplan-Meier survival analysis revealed that the expression of CapG in tumor samples was not significantly associated with disease-free survival time. In conclusion, CapG was overexpressed in CRC and was associated with tumor progression; therefore, it may be a useful prognostic biomarker and therapeutic target in CRC.

Introduction

Colorectal carcinoma (CRC) is the third most common type of cancer worldwide; 40-50% of newly diagnosed patients have already progressed to metastasis and are therefore resistant to conventional therapy, with an increased chance of recurrence (1,2). Despite advances in CRC therapy, the prognosis of patients with metastatic CRC (mCRC) remains poor, with a median overall survival (OS) time of 18-21 months (3). Thus, the major risk factor for patients with CRC is the development of metastasis.

Understanding the molecular mechanisms that drive CRC progression and metastatic processes may facilitate the development of better treatment strategies to improve patient prognosis and disease management, and aid in the identification of novel molecular prognostic factors and therapeutic targets. One candidate molecule with potential as a prognostic marker or therapeutic target is macrophage-capping protein (CapG). CapG is a ubiquitous actin-binding protein of the gelsolin/villin superfamily that is associated with cell motility (4). A previous study demonstrated that bone marrow-derived macrophages from CapG-deficient mice exhibited distinct motility defects and the inhibition of receptor-mediated ruffling, suggesting that CapG is associated with motility (4). Furthermore, the overexpression of CapG has been detected in a range of types of cancer, including pancreatic, breast and ovarian carcinoma, in which the contribution of CapG expression to cancer cell metastatic behavior is validated (5). However, there is limited information regarding the role of CapG in CRC.

Therefore, the present study investigated the role of CapG in CRC development and progression, with potential novel insights for CRC diagnosis, treatment and prognosis.

Materials and methods

Human tissue sample collection. Between October 2014 to March 2015, fresh tissues were obtained from 84 patients with CRC (49 males, 35 females) that underwent CRC resection without neoadjuvant treatment at the Zhongnan Hospital of Wuhan University (Wuhan, China). The mean age was 59.3 (range, 29-85) years. the study was approved by the Zhongnan Hospital of Wuhan University Ethics Committee. Informed, written consent was obtained from all participants in the study. The neoplastic tissues were collected from 84 patients, whereas additional non-neoplastic epithelial tissue samples (~5 cm from the border of the main tumor lesion) were collected from 19 of the patients. The tissue samples were formalin-fixed and paraffin-embedded. Data regarding the clinicopathological features of the patients, including sex, age, tumor location, tumor differentiation, lymph node metastasis (LNM) status and clinical stage

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determined according to the Dukes system (6) for CRC staging were extracted from patient records. Patients that had received prior treatment or that exhibited metaplasia, dysplasia or atypical hyperplasia were excluded from the study.

Immunohistochemistry (IHC). For IHC, formalin-fixed, paraffin-embedded CRC and non-neoplastic epithelial tissues were cut into 4-μm sections. The sections were deparaffinized in xylene and rehydrated in a descending series of ethanol concentrations. For antigen retrieval, sections were immersed in antigen-unmasking solution (BOND Epitope Retrieval 1; Leica Microsystems, Inc., Buffalo Grove, IL, United States; cat. no. AR9961, pH 6.0, 10 min, 100˚C) and boiled in a microwave oven for 15 sec. Tissue sections were incubated with anti-CapG antibodies (no. 10194-1-AP; dilution, 1:500; ProteinTech Group, Inc., Chicago, IL, USA) at room temperature for 60 min. A S-P immunohistochemical kit (Fuzhou Maixin Biological Technology, Ltd., Fuzhou, China) was then applied according to the manufacturer's protocol. Immunohistochemical reactions were developed with 3,3'-diaminobenzidine tetrahydrochloride (Fuzhou Maixin Biological Technology, Ltd.) and counterstained with hematoxylin for 30 sec, prior to mounting.

Immunostaining was blindly evaluated by two independent experienced pathologists in an effort to provide a consensus on staining patterns. The number of positive cells per core were counted at x200 and x400 magnification. A total of 5 cores were taken per sample and the diameter of each core was 4 cm. The grade of each section was judged by the staining intensity and percentage of positive cells. The scores for staining intensity were 0 (no staining), 1 (weak staining), 2 (moderate staining) or 3 (strong staining); the scores for the percentage of positive cells were 0 (<5%), 1 (≥5% and <25%), 2 (≥25% and <50%), 3 (≥50% and <75%) or 4 (≥75%). The combined IHC score was the staining intensity score multiplied by the positive percentage score: Negative (combined score, ≤1) was designated with '-', weak positive (combined score, 2-4) with '+', moderate positive (combined score, 6-8) with '++' and strong positive (combined score, 9-12) with '+++', as per a previously described method (7).

Cell culture and small interfering (si)RNA. HCT116 CRC cells were obtained from the Scientific Research Center of the Zhongnan Hospital of Wuhan University and cultured in HyClone RPMI-1640 medium (GE Healthcare Life Sciences, Logan, UT, USA) with 10% fetal bovine serum (FBS; Zhejiang Tianhang Biotechnology Co., Ltd., Luohe, China). The cells were incubated at 37˚C in a humidified atmosphere with 5% CO_2 throughout the study. siRNA oligo duplexes were produced by Invitrogen (Thermo Fisher Scientific, Inc.). The target sequences for CapG-siRNA were forward, 5'-GUGUGG AGUCAGCAUUUCAdTdT-3' and reverse, 3'-dTdT CACACC UCAGUCGUAAAGU-5'; and the negative control sequences were forward, 5'-UUCUCCGAACGUGUCACGUdTdT-3' and reverse, 5'-ACGUGACACGUUCGGAGAAdTdT-3'. The CapG or negative control siRNA was transfected into HCT116 cells with Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following incubation for 48 h, the cells were harvested and the efficacy of RNA interference (RNAi) was confirmed by western blotting.

Western blotting. The HCT116 cells were lysed with 100-300 μl RIPA buffer supplemented with protease inhibitors (Roche Diagnostics, Basel, Switzerland). The protein concentration was measured with the BCA Protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). From each sample, 20 μg total protein per lane was separated by SDS-PAGE (10% gel). Proteins were electroblotted onto a polyvinylidene fluoride membrane and blocked overnight in 0.05% Tween-20 in PBS with 5% dried skimmed milk at 4˚C. Primary antibodies against CapG (no. 10194-1-AP; dilution, 1:1,000; ProteinTech Group, Inc.) and GAPDH (no. BM1985; 1:1,000; Wuhan Boster Biological Technology Ltd., Wuhan, China) were incubated with the membrane for 1 h at room temperature. Membranes were then washed with 0.05% Tween-20 in PBS and incubated with secondary antibodies, including peroxidase-conjugated anti-rabbit (no. sc2357; 1:3,000; against CapG antibodies) and anti-mouse (no. sc516142; 1:3,000; against GAPDH antibodies) (both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibodies, for 45 min at room temperature. The membranes were visualized using an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology, Inc.). GAPDH was used as a control for protein loading. Quantification of the intensity of immunoblots was performed by Bio-Rad Quantity One software (version 4.1; Bio-Rad Laboratories, Inc.).

Wound healing assay. HCT116 cells were treated with siRNA as previously described. Following incubation for 24 h, the cells were removed by trypsinization, counted and plated at $4x10^5$ cells/ml in 6-well plates. Cells were incubated overnight to yield confluent monolayers. A wound in the monolayer was produced with a pipette tip and images were captured at 0 and 24 h after wounding. Wound closure (%) was determined as the wound width at 24 h relative to the width at 0 h. Experiments were performed in triplicate and repeated ≥3 times.

Transwell migration assay. In vitro tumor cell migration was measured using Transwell chambers without matrigel. (BD Biosciences, Bedford, MA, USA) according to the manufacturer's protocol. In brief, $2x10^5$ cells with/without small interfering RNA in RPMI-1640 medium with 2% FBS were plated in the upper chamber, with RPMI-1640 medium containing 10% FBS in the bottom chamber. The cells were incubated for 24 h. The cells on the bottom surface were fixed in 4% formalin for 15 min at room temperate, washed with PBS twice, stained with 0.1% crystal violet for 15 min at room temperate, and were counted. Cell counting was manually performed in 5 areas per membrane with an optical microscope at x200 magnification.

Statistical analysis. The CRC mRNA expression profiles GSE14333 and GSE39582 were downloaded from the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo). Disease-free survival (DFS) analyses were performed with the Kaplan-Meier method and the results were compared by the log-rank test. The median (for GSE14333, the median is 8.83; for GSE39582, the median is 8.87) was used as the cut-off value in the Kaplan-Meier analysis to define the low

Table I. CapG IHC scores in CRC tissue compared with non-tumor tissue samples.

	CapG IHC scores						
Tissue type	Total	-	+	++	+++	χ^2	P-value
CRC tissue	19	3	2	10	4	20.618	<0.001
Non-tumor tissue	19	14	5	0	0		

CapG, macrophage-capping protein; IHC, immunohistochemistry; CRC, colorectal carcinoma.

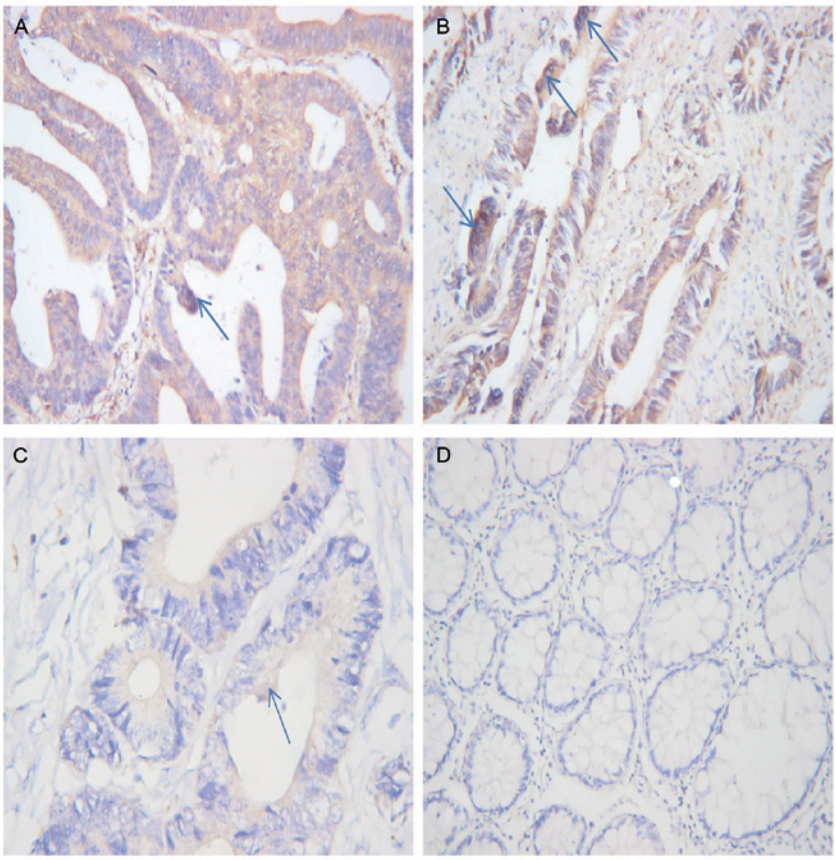

Figure 1. Expression of CapG protein is increased in CRC tissue relative to non-tumor tissue samples. Representative images of CRC tissue samples with (A) high CapG expression (magnification, x200), (B) moderate CapG expression (magnification, x200) and (C) low CapG expression (magnification, x400). (D) Normal colorectal mucosa cells with negative CapG expression (magnification, x200). The arrows indicate the distribution of CapG in the cytoplasm and nucleus. CapG, macrophage-capping protein; CRC, colorectal carcinoma.

and high expression groups. Associations between CapG expression and clinicopathological parameters were assessed by the χ^2 test. The wound healing and Transwell assay results were analyzed by the t-test. Data are expressed as the mean (n=3) ± standard deviation. P<0.05 was considered to represent a statistically significant difference. Statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA).

Results

CapG expression is higher in CRC tissue than in non-tumor tissue samples. The IHC results revealed that CapG expression in the CRC tissue was higher than in the non-tumor tissue. The positive CapG expression rate was 84% (16/19) for CRC tissue and 26% (5/19) for non-tumor tissue. Compared with the non-tumor tissue, CRC tissue exhibited a significantly increased rate of immunoreactivity for CapG (P<0.001; Table I). CapG positivity was identified primarily in the cytoplasm and nucleus of the CRC cells (Fig. 1).

CapG overexpression is associated with risk-associated prognostic factors and the progression of CRC. Potential associations between CapG expression and clinicopathological parameters are summarized in Table II. The results indicated that the positive expression of CapG was significantly associated with tumor site, LNM status, tumor differentiation and clinical stage (P=0.021, P=0.036, P=0.012 and P=0.009, respectively); however, there was no significant difference in expression associated with sex or age (P=0.366 and P=0.789,

Table II. Association of the clinical features of patients with CRC with CapG IHC scores.

Clinical features	CapG IHC scores					χ^2	P-value
	Total	-	+	++	+++		
All patients	84	4	22	31	27		
Sex						3.17	0.366
Male	49	1	12	21	15		
Female	35	3	10	10	12		
Age						1.05	0.789
≥65	30	2	9	11	8		
<65	54	2	13	20	19		
Location						14.96	0.021
Rectum	28	2	14	7	5		
Left colon	32	1	3	15	13		
Right colon	24	1	5	9	9		
Lymph node metastasis						8.53	0.036
Yes	34	1	6	10	17		
No	50	3	16	21	10		
Tumor differentiation						16.43	0.012
High	15	2	7	4	2		
Moderate	52	1	15	21	15		
Low	17	1	0	6	10		
Stage						11.64	0.009
A/B	41	4	15	14	8		
C/D	43	0	7	17	19		

CRC, colorectal carcinoma; CapG, macrophage-capping protein; IHC, immunohistochemistry.

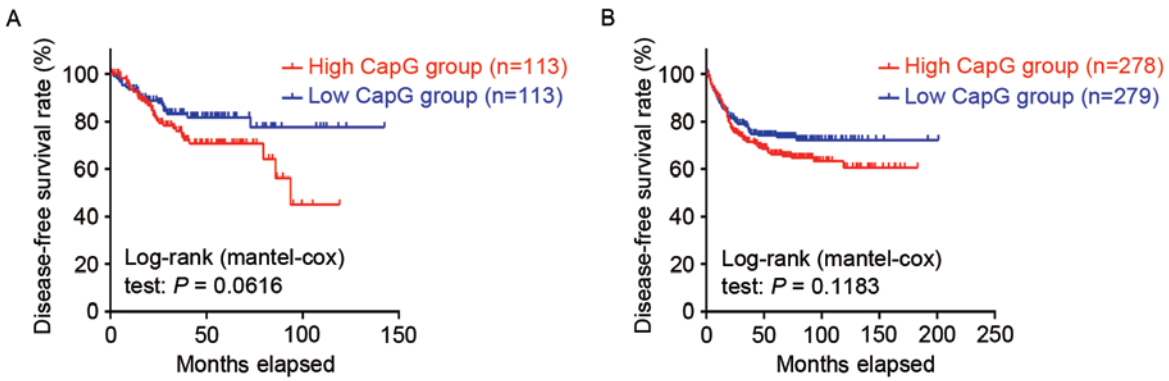

Figure 2. Increased expression of CapG is not significantly associated with a poor prognosis in patients with CRC. (A) CapG mRNA levels were not associated with the DFS time in patients with CRC in the dataset GSE14333. (B) CapG mRNA levels were not associated with the DFS time in patients with CRC in the dataset GSE39582. CapG, macrophage-capping protein; CRC, colorectal carcinoma; DFS, disease-free survival.

respectively). These results may suggest that CapG functions as an oncogene in CRC, and CapG may represent a novel prognostic factor for CRC following curative colorectal resection. However, Kaplan-Meier survival analysis of the GSE14333 and GSE39582 expression profiles demonstrated that DFS time did not differ significantly between the patients with CRC with tumors with high and low CapG expression levels (GSE14333; P=0.0616, Fig. 2A; GSE39582, P=0.1183, Fig. 2B, respectively).

Reduction of CapG significantly inhibits the in vitro motility of HCT116 cells. RNAi was effective in reducing the expression level of CapG protein in HCT116 cells (Fig 3A). CapG levels were diminished in HCT116 cells at 48 h after transfection with CapG-specific siRNA compared with cells transfected with the control siRNA.

A migration assay and an *in vitro* wound-healing assay were used to assess the effect of CapG knockdown on cell motility. In the wound healing assay, a wound in a monolayer of cells

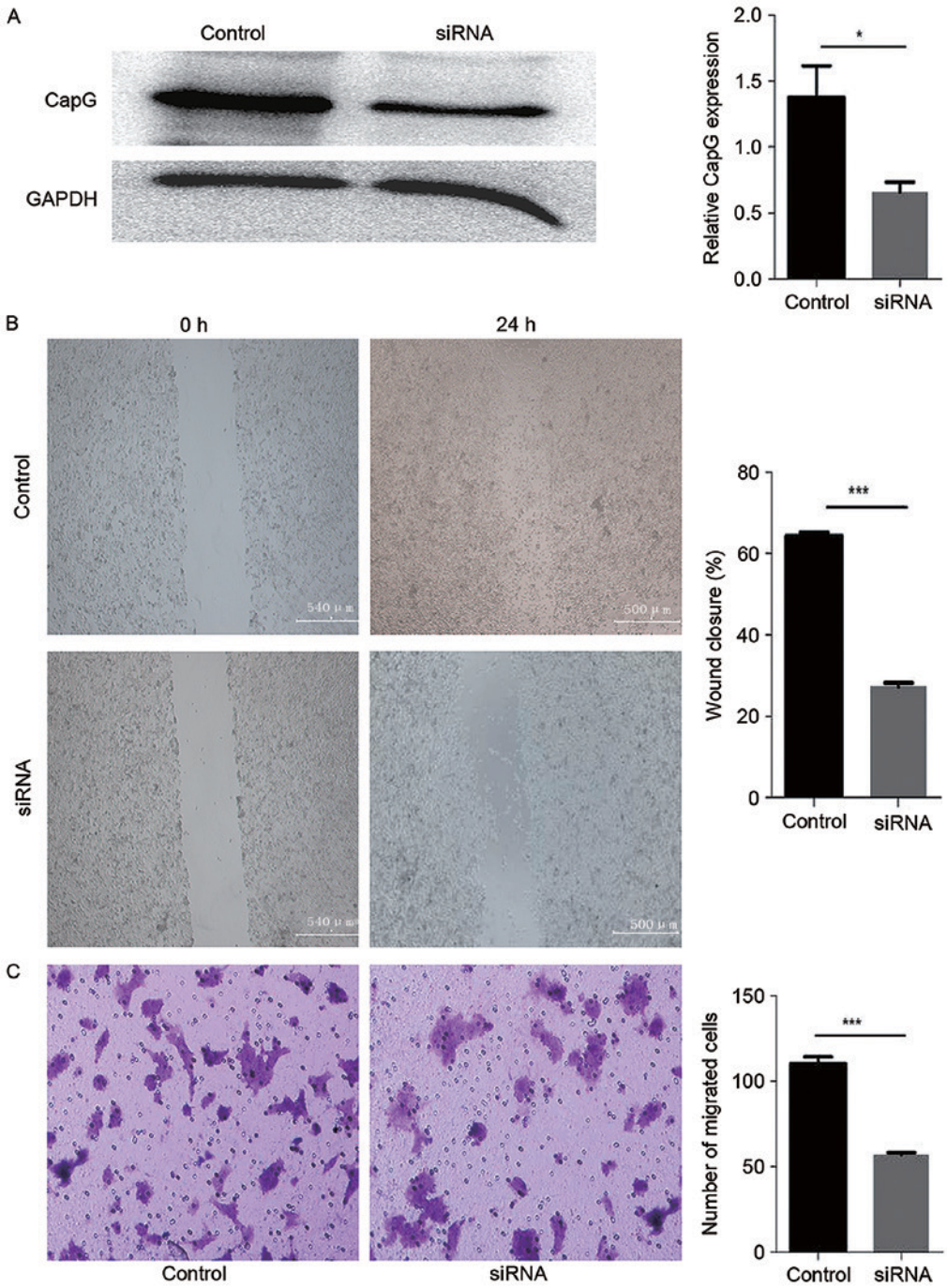

Figure 3. Functional assays of CapG in CRC cells. (A) CRC cells transfected with CapG-specific siRNA (siRNA) exhibited a reduced expression of CapG protein compared with corresponding cells transfected with a scrambled siRNA (control). (B) Knockdown of CapG by RNA interference inhibited wound healing. (C) Knockdown of CapG by RNA interference inhibited cell migration (magnification, x200). *P<0.05, ***P<0.001. CapG, macrophage-capping protein; CRC, colorectal carcinoma; siRNA, small interfering RNA.

transfected with the control siRNA exhibited >65% wound closure. In contrast, cells transfected with the CapG-targeting siRNA exhibited <27% wound closure. This demonstrated that the capacity for cell migration significantly decreased when CapG expression was suppressed (P<0.001; Fig. 3B).

The result of the cell migration assay was consistent with the result of the wound healing assay. Transfection of the HCT116 cells with the siRNA against CapG resulted in a reduced extent of motility compared with corresponding cells treated with control siRNA, as demonstrated by the reduced rate of migration (P<0.001; Fig. 3C).

Discussion

CRC is the third most common type of cancer worldwide (8). The 5-year survival rate is ~90% for patients with early stage CRC, but decreases to <10% in patients with distant metastasis (9). Therefore, it is necessary to identify mCRC risk-associated biomarkers.

CapG, a 348-amino acid protein, belongs to the actin-binding protein family, is ubiquitously expressed in normal tissue, with particular abundance in macrophages (10,11), and is associated with cell signaling, receptor-mediated membrane ruffling,

phagocytosis and motility (12,13). It has been reported to modulate the motility of cells by interacting differentially with the actin cytoskeleton (14). CapG was originally isolated from the cytoplasm of alveolar macrophages and has been demonstrated to be involved in the control of actin-based cell motility and membrane ruffling (phagocytosis) of non-muscle cells; it may also function as a nuclear actin-binding protein to prevent nuclear actin from polymerizing and maintain it in a monomeric globular or short oligomeric form (14). Alteration to CapG can change the cell morphology and reduce motility (15), particularly important features of cancer cells during invasion and metastasis.

It has been reported that CapG is associated with invasion and metastasis in various types of malignancy (15-17). However, to the best of our knowledge, the effect of CapG in primary CRC was not investigated prior to the present study. In the present study, the expression and function of CapG in CRC were investigated, and the results demonstrated that the expression level of CapG protein in CRC tissue was significantly higher than in non-tumor tissue. This observation was consistent with the results of studies regarding other types of cancer, including oral (18), pancreatic (19), ovarian (20-22) and breast cancer (23). In the present study, the positive expression of CapG in the cytoplasm and nucleus was significantly associated with CRC location, differentiation, LMN status and clinical stage. Other clinical studies of nasopharyngeal carcinoma (24), non-small cell lung cancer (25) and cholangiocarcinoma (26) concluded that high CapG expression was associated with poor differentiation and clinical stage, and that the patients with CapG-positive tumors exhibited a worse prognosis. Ichikawa *et al* (15) performed two-dimensional gel electrophoresis to obtain protein expression profiles for 3,228 proteins, and identified that CapG was upregulated in the tumor tissues of patients with LNM. This is consistent with the result of the present study. Furthermore, although there was no identified statistical significance, the expression of CapG trended towards an association with DFS time, which implies that CapG may be useful in establishing a prognosis in CRC.

Clinical studies of pancreatic ductal and lung adenocarcinoma have also demonstrated that high CapG expression was associated with an increased tumor size (19,25). Morofuji *et al* studied the proteomic profile of cholangiocarcinoma and identified CapG expression as a novel biomarker for predicting the response to gemcitabine treatment, and as a prognostic indicator in cholangiocarcinoma (26). However, further validation studies are required to establish whether CapG may exhibit similar functions in CRC.

The IHC results revealed that the positive expression of CapG in the cytoplasm and nucleus was significantly associated with the location of the CRC tumor; the highest expression of CapG was identified in tumors from the left side of the colon. Compared with CRC from the right side of the colon, left-sided colon cancer is more likely to metastasize (27,28). An additional study identified that CRC that metastasized to the liver had a higher expression of CapG (29). This result may support the conclusion from the present study that CapG in CRC was significantly associated with the tumor site, as CRC from different locations is associated with different rates of liver metastasis (28).

When CapG expression was suppressed in the present study, the motility of CRC cells was reduced. In the migration and *in vitro* wound-healing assays, cell migration was significantly inhibited following the transfection of HCT116 cells with siRNA against CapG. This suggests that CapG contributes to the motility of CRC cells. Other studies have reported that the knockdown of CapG in hepatocellular carcinoma (17) and pancreatic cancer (19) cells can attenuate cancer cell invasion, motility and aggression. However, Watari *et al* (30) identified that CapG may act as a tumor suppressor in stomach cancer, lung cancer and melanoma. Therefore, the role of CapG in tumor cells may depend on the cell type.

The nuclear import of CapG is energy dependent and requires the cytosolic receptor importin β (31). It has been reported that the overexpression of nuclear CapG, but not cytoplasmic CapG, predominantly contributes to cell invasion (31). Whether cytoplasmic or nuclear CapG expression promote cell invasion specifically in CRC may require further study.

Taken together, the results of the present study demonstrated that CapG expression was increased in CRC tissue and associated with poor prognostic risk factors. The observation that the downregulation of CapG in CRC cells diminished their motility may imply the involvement of CapG in the motility, and consequently the dissemination, of CRC cells. These findings may provide novel insights to understanding the molecular mechanisms of CRC metastasis, and CapG may be a potential biomarker for predicting the prognosis of CRC.

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