Polymorphisms of *Survivin* and Its Protein Expression Are Associated with Colorectal Cancer Susceptibility in Chinese Population

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To investigate the association of *survivin* −31G/C, −141G/C, and −241T/C polymorphisms with colorectal cancer (CRC) susceptibility and explore the mechanisms of the *survivin* polymorphism in CRC development. A case–control study was conducted of 275 CRC cases and 270 healthy controls. Polymorphisms of *survivin* −31G/C, −141G/C, and −241T/C were genotyped by polymerase chain reaction–restriction fragment length polymorphism. Survivin and Ki-67 expression was analyzed by immunohistochemistry by the Envision technique for the paraffin sections of 152 CRC. It showed that the −31G/C genotype and allele distribution were significantly different between the CRC cases and controls. The −31CC genotype and −31C allele were overrepresented among the CRC cases. Compared with the CC genotype, the GC and GG genotypes had a significantly decreased risk of CRC ($p=0.015$). Survivin and Ki-67 expression of patients with the CC genotype was significantly higher than the patients with the GC and GG genotypes. In addition, a significantly positive correlation was found between expression of Survivin and Ki-67. There were no significant difference of the −141G/C and −241T/C polymorphism distributions among cases and controls. Survivin 31G/C may adjust the Survivin expression, and it might contribute to a risk of developing CRC.

Introduction

DEFECTS IN THE apoptosis signaling pathway that are common in cancer cells are thought to play an important role in tumor initiation, progression, and metastasis. The inhibitor of apoptosis proteins (IAPs) is a family of antiapoptotic proteins that inhibit initiator (caspase-9) and effector caspases (caspase-3 and caspase-7) to prevent apoptosis (Thompson, 1995; Raff, 1998). Survivin, a member of the IAP family, plays a key role in modulating processes, such as cell division, tissue patterning, cytokine activation, and activation of various cell signaling pathways (Altieri, 2003; Pennati *et al.*, 2008). Experimental data have implicated the role of *survivin* in promotion of cell proliferation as well as inhibition of apoptosis in cancer cells (Ambrosini *et al.*, 1998). Highly expressed Survivin has been detected in many types of human cancers, including colorectal cancer (CRC) (Gazouli *et al.*, 2009; Srivastava *et al.*, 2012). Survivin overexpression was associated with neoplasia and metastasis, suggesting that it might be used as one of the most important diagnostic and prognosis markers for many types of cancers (Altieri, 2003; Montorsi *et al.*, 2007; Li *et al.*, 2008). Polymorphisms in the *survivin* may influence Survivin production or activity, thereby modulating susceptibility to cancer (Jang *et al.*, 2008). As of yet, around 199 single-nucleotide polymorphisms (SNPs) have been identified in the *survivin* gene in humans (Srivastava *et al.*, 2012).

CRC, one of the frequent cancers worldwide, arises as a result of the accumulation of genetic errors, many of which affect the control of apoptosis. Effective chemoprevention strategies for CRC must rectify these genetic defects. Ki-67, a nuclear antigen, is a marker of cell growth. It is expressed during all phases of the cell cycle (G1, S, G2, and mitosis), except phase G0. In recent studies, important correlation was found between Ki-67 and the growth, invasion, metastasis, and prognosis of cancer (Gzy Zew Ska *et al.*, 2004). In this study, our aim was to investigate whether the −31G/C, −141G/C, and −241T/C SNPs of the human *survivin* could represent a risk factor for CRC development and explore the expression of Survivin and Ki-67 in CRC patients with different genotypes.

Materials and Methods

Study population

This case–control study consisted of 275 CRC patients and 270 healthy controls. All subjects were unrelated Han nationality living in Luzhou City, Sichuan Province, and

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TABLE 1. CHARACTERISTICS OF THE STUDY POPULATION

Variables	CRC patients, n = 275(%)	Controls, n = 270(%)	χ^2	p
Age				
≥ 60	130 (47.3)	125 (46.3)	0.052	0.819
< 60	145 (52.7)	145 (53.7)		
Sex				
Male	152 (55.3)	143 (53.0)	0.293	0.588
Female	123 (44.7)	127 (47.0)		
Smoking status				
Smokers	107 (38.9)	126 (46.7)	3.350	0.067
Nonsmokers	168 (61.1)	144 (53.3)		
Alcohol use				
Drinkers	132 (48.0)	122 (45.2)	0.434	0.510
Nondrinkers	143 (52.0)	148 (54.8)		

CRC, colorectal cancer.

surrounding regions. CRC patients were found with newly diagnosed disease and were recruited from Department of Oncology, the First Affiliated Hospital of Luzhou Medical College, between July 2009 and March 2011. The patients were histological confirmed by the local diagnosing pathologists. Controls were randomly selected from 3200 individuals who participated in a healthy checkup during the same period as the cases were recruited. These control subjects were matched with the patients in terms of age and sex, excluding those with medical history of surgery or chronic disease. Approval of the study was given by the ethics committee of Chinese Human Genome. Each subject was personally face-to-face interviewed by trained interviewers for information on demographic data as well as related risk factors such as tobacco smoking, alcohol drinking, and medical history of viral hepatitis type. After interview, ~5-mL venous blood sample was collected. DNA was extracted from the peripheral blood with the phenolic chloroform method and frozen at −20°C until tested. Paraffin blocks were collected from the CRC patient postoperative sample.

Table 1 summarizes the characteristics of the 275 CRC patients and 270 healthy control subjects included in this study. The median age of the patients was 54 years (range 35–82 years) and for the controls was 56 years (range 32–80 years). No significant differences were identified between the cases and controls for the age, sex, smoking, and alcohol consumption status. This suggested that the matching

based on these two variables was adequate. The criteria for exclusion of the study subjects were as follows: the subjects with recurring CRC, history of cancers, other serious diseases, genetic relationship, or the subjects refusing to inquisition, and no detailed personal and clinicopathological data.

Genotypes

The −31G/C, −141G/C, and −241T/C genotypes of *survivin* were determined by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). The PCR primers were designed by Primer design software primer3 and provided by Shanghai Sangon Biological Engineering Company. The primers for PCR, the restriction enzymes (MBI Fermentas), and the fragments length after digestion are shown in Table 2. Amplification was carried out on Applied Bio-systems AB (USA). The annealing temperature was 53°C, 55°C, and 56°C for −31G/C, −141G/C, and −241T/C, respectively. After digested by restriction enzymes, the digested products were analyzed on 8% nondenaturing polyacrylamide gel electrophoresis followed by silver staining. The genotyping method was confirmed by the DNA sequencing analysis.

Immunohistochemistry

Paraffin sections (3 μm) were dried, deparaffinized, and rehydrated in graded alcohol to water. Heat-mediated antigen retrieval was performed using pressure cooker treatment for 5 min in the EDTA buffer (pH 8.0). Endogenous peroxide activity was quenched by applying 3% hydrogen peroxide for 10 min. The slides were incubated for 40 min at 37°C with a primary antibody for Survivin (rabbit polyclonal Survivin antibody, 1:100; Bio-World) and Ki-67 (clone: MIB-1, 1:200; Dako). After washing, the tissue sections were incubated in the second antibodies (Envision™ and HRP rabbit/mouse; Dako) for 20 min at 37°C. In the negative controls, the primary antibody was omitted. The reaction products were visualized by DAB.

Expression of Survivin was determined in the nucleus and in the cytoplasm by assessing semiquantitatively the percentage of marked tumor cells and the staining intensity. Score for percentage of positive cells is as follows: 1, 1%–10% positive cells; 2, 11%–50%; 3, 51%–80%; and 4, >80% positive cells. Staining intensity was scored as 1, weak; 2, moderate; and 3, intensive. Scores for the percentage of positive cells

TABLE 2. POLYMERASE CHAIN REACTION–RESTRICTION FRAGMENT LENGTH POLYMORPHISM-BASED ASSAY OF SURVIVIN SNPS

SNP	Position	Sequence of primer	Enzyme	Interpretation (bp)
rs9904341	−31	5-GACTACAACTCCCGGCACAC-3 5-TGTAGAGATGCGGTGGTCCT-3	*Eco*O109I	CC: 226 GC: 226, 134, 92 GG:134, 92
rs17882312	−141	5-GATTACAGGCGTGAGCCACT-3 5-TCAAATCTGGCGGTTAATGG-3	*Ban*I	GG:162, 104 GC: 266, 162, 104 CC: 266
rs17878467	−241	5-CTCAGCCTCCCGAGTAGTTG-3 5-TCAAATCTGGCGGTTAATGG-3	*Hae*I	CC: 220, 202 TC: 422, 220, 202 TT: 422

SNP, single-nucleotide polymorphism.

TABLE 3. FREQUENCY DISTRIBUTIONS OF SURVIVIN POLYMORPHISMS AMONG CASES AND CONTROLS

Polymorphisms	Cases, n = 275(%)	Controls, n = 270(%)	OR (95% CI)[a]	p
−31G/C Genotype				
CC	110 (40.0)	77 (28.5)	1.000	0.015
GC	123 (44.7)	138 (51.1)	0.618 (0.420–0.907)	
GG	42 (15.3)	55 (20.4)	0.519 (0.312–0.865)	
Allele frequency				
C	343 (62.4)	292 (54.1)	1.000	0.006
G	207 (37.6)	248 (45.9)	0.711 (0.558–0.905)	
−241T/C genotype				
CC	166 (60.4)	165 (61.1)	1.000	0.919
TC	92 (33.4)	88 (32.6)	1.083 (0.732–1.602)	
TT	17 (6.2)	17 (6.3)	1.084 (0.509–2.308)	
Allele frequency				
C	424 (77.1)	418 (77.2)	1.000	0.901
T	126 (22.9)	122 (22.8)	1.018 (0.767–1.352)	

[a]Adjusted for sex, age, smoking, and alcohol consumption status.
OR, odds ratios; CI, confidence intervals.

and scores for expression intensities were multiplied to calculate an immunoreactivity score. The score ≤ 3 considered negative (−) and 4–12 considered positive (+) (Taubert et al., 2010). Ki-67 was evaluated by counting the number of positive nuclei in 500 tumor cells, and the percentage was calculated as the Ki-67-labeling index. Ki-67 negativity was defined as $\leq 10\%$ (−), and positivity was defined as $>10\%$ (+) (Al Gizawy et al., 2011).

Statistical analysis

Genotypes were tested for the Hardy–Weinberg equilibrium test. The numeration data groups were compared using the chi-square test; relationship between Survivin and Ki-67 was assessed using the Spearman's rank correlation analysis. The odds ratios and 95% confidence intervals were calculated by multivariate logistic regression model test, adjusted

TABLE 4. THE ASSOCIATIONS OF THE CLINICOPATHOLOGIC PARAMETERS OF COLORECTAL CANCER
AND THE SURVIVIN 31G/C SINGLE-NUCLEOTIDE POLYMORPHISMS

Clinicopathological parameters	−31G/C genotype			χ^2	p
	CC n = 110 (%)	GC n = 123 (%)	GG n = 42 (%)		
Location				3.209	0.201
Rectum	70 (63.6)	65 (52.8)	22 (52.4)		
Colon	40 (36.4)	58 (47.2)	20 (47.6)		
Gross type				1.574	0.455
Ulcerated	61 (55.5)	72 (58.5)	28 (66.7)		
Elevated type	49 (44.5)	51 (41.5)	14 (33.3)		
Tumor size (cm)				1.296	0.523
≥ 3	72 (65.5)	89 (72.4)	29 (69.0)		
<3	38 (34.5)	34 (27.6)	13 (31.0)		
Histologic type				3.558	0.169
Adenocarcinoma	101 (91.8)	108 (87.8)	34 (81.0)		
Mucinous adenocarcinoma	9 (8.2)	15 (12.2)	8 (19.0)		
Depth of invasion				1.091	0.580
Within serosa	66 (60.0)	78 (63.4)	29 (69.0)		
Serosa and beyond	44 (40.0)	45 (36.6)	13 (31.0)		
Lymph node metastasis				0.225	0.893
Yes	49 (44.5)	51 (41.5)	18 (42.9)		
No	61 (55.5)	72 (58.5)	24 (57.1)		
TNM stage				1.812	0.404
I + II	63 (57.3)	81 (65.9)	26 (61.9)		
III + IV	47 (42.7)	42 (34.1)	16 (38.1)		
Differentiation (adenocarcinoma)				3.343	0.188
Well	49 (44.5)	69 (56.1)	23 (54.8)		
Poor	61 (55.5)	54 (43.9)	19 (45.2)		

for sex, age, smoking, and alcohol consumption status. For all hypothesis tests, a two-tailed significance level of $p < 0.05$ was considered statistically significant. All the data were analyzed using the SPSS 11.5 statistical package.

Results

Survivin gene polymorphisms of the subjects

The genotype distributions of $-31G/C$ and $-241T/C$ polymorphisms among the controls and the cases were in the Hardy–Weinberg equilibrium ($-31G/C$: $\chi^2 = 0.128$, $p = 0.720$; $-241T/C$: $\chi^2 = 1.989$, $p = 0.158$). Genotype and allele frequencies of $-31G/C$ and $-241T/C$ among the cases and controls are summarized in Table 3. The frequencies of the CC, GC and GG genotypes for the $-31G/C$ polymorphism were 40.0%, 44.7%, and 15.3% among cases and 28.5%, 51.1%, and 20.4% among controls, respectively. It showed significant differences in the $-31G/C$ genotypic distributions ($\chi^2 = 8.383$, $p = 0.015$). The $-31C$ allele was over-represented among the CRC cases ($\chi^2 = 7.699$, $p = 0.006$). Adjusted for sex, age, smoking, and alcohol consumption status, the risks of CRC for individuals carrying the GC and GG genotypes were decreased, and they were 0.618-fold and 0.519-fold compared with the individuals carrying the CC genotype. The $-31CC$ genotype may increase the risk of CRC.

Comparisons between the case and control groups showed no significant differences in the $-241T/C$ genotypic distributions. In this study, no polymorphism of $-141G/C$ was found, and the genotype was CC.

The associations of clinicopathologic parameters of CRC and $-31G/C$ polymorphisms

The association of the genotypic distribution of the $-31G/C$ polymorphism and clinicopathologic features of CRC was analyzed. Results showed that the location of tumor, gross type, tumor size, histologic type, depth of invasion, differentiation degree, lymph node metastasis, and TNM stage were not associated with the $-31G/C$ polymorphism. The data of the $-31G/C$ genotype and clinicopathologic parameters are summarized in Table 4.

The expression of Survivin and Ki-67 in CRC with various $-31G/C$ genotypes

Expression of Survivin was determined most in the nucleus, small part in the cytoplasm. The Ki-67 immunostaining was confined to the nucleus. The positive cells of Survivin and Ki-67 decreased gradually from CC to GC, GG (Fig. 1). The expression of Survivin and Ki-67 was associated with the $-31G/C$ polymorphism (Table 5, $p < 0.05$). Survivin and Ki-67 expression of patients with the CC genotype was significantly higher than that in patients with the GC and GG genotypes ($p < 0.05$). There were no significant differences of Survivin and Ki-67 expression between patients with the GC and GG genotype.
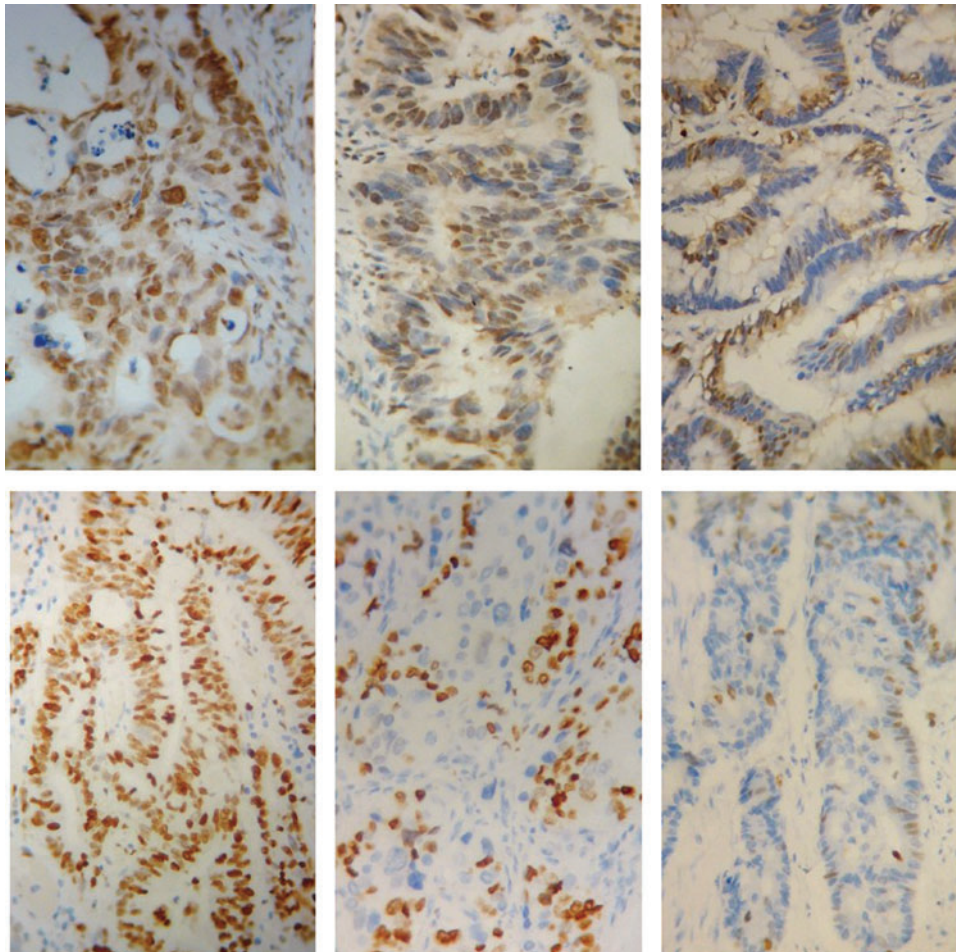

FIG. 1. Immunohistochemical staining for Survivin (above row) and Ki-67 (following row) in colorectal cancer with the CC genotype (left column), GC genotype (center column), and GG genotype (right column) ($\times 400$). Color images available online at www .liebertpub.com/dna

Table 5. The Relationship of the Survivin −31G/C Polymorphism and Survivin,
Ki-67 in Colorectal Cancer Patients

Polymorphism	Survivin, n (%)				Ki-67, n (%)			
	+	−	χ^2	p	+	−	χ^2	p
CC	54 (90.0)	6 (10.0)	5.356[a]	0.021[a]	59 (98.3)	1 (1.7)	5.758[a]	0.016[a]
GC	45 (73.8)	16 (26.2)	0.210[b]	0.646[b]	53 (86.9)	8 (13.1)	0.154[b]	0.695[b]
GG	22 (71.0)	9 (29.0)	5.378[c]	0.020[c]	26 (83.9)	5 (16.1)	6.942[c]	0.008[c]
			6.697[d]	0.035[d]			6.970[d]	0.031[d]

+, Survivin positivity was defined as score 4–12; Ki-67 positivity was defined as >10%.
−; Survivin negativity was defined as score ≤3; Ki-67 negativity was defined as ≤10%.
[a]Compare with CC and GC groups.
[b]Compare with GC and GG groups.
[c]Compare with GG and CC groups.
[d]Compare with all of three groups.

After Spearman's rank correlation analysis (Table 6), the expression of Survivin in CRC was positively correlated with the expression of Ki-67 ($r = 0.178$, $p = 0.029$).

Discussion

CRC is characterized by the partial suppression of apoptosis, which in turn gives tumor a selective advantage for survival and can cause current chemotherapy approaches to be ineffective (Li and Brattain, 2006). Survivin promotes tumor development and progression by inhibiting apoptosis and increasing cell proliferation (Altieri, 2008). In the above case–control study, we evaluated the relationship between the polymorphisms of −31G/C, −141G/C, and −241T/C and CRC risk in a Chinese Han population. In addition, Survivin and Ki-67 expression had been analyzed.

Results showed that the −241T/C polymorphism was not associated with the susceptibility of CRC, and there was no −141G/C polymorphism of *survivin* in our study. However, the −31CC genotype could increase the risk of CRC. We also showed that the −31G/C SNP was not associated with any clinicopathologic features of CRC, such as the location of tumor, depth of invasion, tumor size, histologic type, lymph node metastasis, and TNM stage. For the clinicopathologic features of CRC, inconsistent with our results, Gazouli *et al.* (2009) showed the −31CC genotype and the −31C allele were significantly more frequent in stage grouping III and IV than in stage grouping I and II. Han *et al.* (2009) showed 31G/C was significantly associated with the age of disease onset of ovarian cancer. It is hard to decipher the reason for the discrepancy. It may be due to the different regions

Table 6. The Relationship Between Survivin
and Ki-67 in Colorectal Cancer Patients

Survivin	Ki-67			
	+	−	r	p
+	113	8		
−	25	6	0.178	0.029

+, Survivin positivity was defined as score 4–12. Ki-67 positivity was defined as >10%.
−; Survivin negativity was defined as score ≤3. Ki-67 negativity was defined as ≤10%.

and malignancy, and the inadequate study design such as a limited sample size or nonrandom sampling should be considered.

In a southern Chinese population, Huang *et al.* (2009) found that the *survivin* gene −31G/C polymorphism is associated with sporadic CRC risk, and the G-variant genotype is the independent protective factor against sporadic CRC. In recent years, some research showed that the −31CC genotype of *survivin* was associated with gastric cancer, ESCC, and oral cancer and may be a risk factor of them in the Chinese population (Cheng *et al.*, 2008; Yang *et al.*, 2009; Weng *et al.*, 2012), as Srivastava *et al.* (2012) suggested an important role of the *survivin* −31G/C polymorphism with cancer risk, especially in the Asian population. However, the mechanism is still unknown. Survivin is involved in the regulation of cell division and survival, two key processes in cancer (Antonacopoulou *et al.*, 2011). In the progress of the CRC, it is not clear whether closer relationship between the *survivin* gene and the cell proliferation.

In the present study, we demonstrated that the expression of Survivin in the cancer tissue of CRC patients with the −31CC genotype was significantly higher than that with the GC and GG genotype, but there were no differences between the GC and GG genotype. Xu *et al.* (2004) reported that the presence of the −31CC genotype was more frequent in cancer cell lines, and mRNA and protein levels of *survivin* increased in these cell lines. In a M Gazouli *et al.* (2009) study, they observed that the *survivin* mRNA level of CRC cases with the −31CC homozygous genotype exhibited ∼1.6-fold higher than the carriers of the −31GG and −31GC genotypes. However, Cheng *et al.* (2008) found that *survivin* mRNA was overexpressed in gastric cancer cases, but with no significant difference in various −31G/C polymorphism genotypes. Our results showed that the Survivin protein expression level of CRC cases with the −31CC genotype was significantly higher than that with the −31GG and −31GC genotypes. This finding confirmed previous studies (Xu *et al.*, 2004; Jang *et al.*, 2008; Yang *et al.*, 2009). The −31G/C polymorphism located at the cell cycle-dependent element/cell cycle homology (CDE/CHR) region repressor elements and conferred aberrant cell cycle-dependent transcription, mediated through the functional disruption of binding at the CDE/CHR repressor motifs (Xu *et al.*, 2004). We speculate that the −31G/C polymorphism may influence *survivin* transcription and translation by modifying the banding motif

of the CDE/CHR repressor. Further studies should be needed to confirm this surmise.

Nucleocytoplasmic localization of Survivin in tumor cells, determined by immunohistochemistry, has been reported to provide prognostic information in several types of cancer (Fortugno et al., 2002; Mohamed et al., 2009). Regarding the Survivin immunostaining, Aune et al. (2011) reported that the nuclear staining was recorded in ovarian carcinomas, and this pattern is obviously related to proliferation, whereas the role of the cytoplasmatic immunoreactivity is not fully clarified. Further, the Survivin indices paralleled those of Ki-67 and PHH3, with a considerable overlap between the malignancy groups (Li, 2005; Li et al., 2005). In our research, the expression of Survivin was determined most in the nucleus, small part in the cytoplasm. Moreover, we found that the expression of Survivin was positively correlated with the expression of Ki-67 in CRC tissues with various −31G/C genotypes. In consistent with the expression of Survivin, the Ki-67 expression of CRC patients with the −31CC genotype was significantly higher than those with the GC and GG genotype. Survivin activates aurora-B kinase (ABK) catalyzing mitosis, and overexpression of Survivin provides activation of ABK, which results in increased mitosis and proliferation (Boman et al., 2007). This effect may be synergistic with Survivin's ability to prevent apoptosis, so both effects would promote tumor growth. Ultimately, both these mechanisms contribute to the exponential increase of proliferative cell populations (Zhang et al., 2010).

Conclusion

The survivin 31G/C polymorphism may adjust the Survivin protein expression, and it might contribute to a risk of developing CRC. The survivin 31G/C polymorphism may be a genetic modifier for CRC prognosis in the Chinese population. Further rigorous studies with larger sample sizes and studies of biological functions of these SNPs are needed to validate the role of the survivin promoter SNPs in the development of CRC.

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Disclosure Statement

No competing financial interests exist.

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