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ORIGINAL PAPER

Evaluation of immunohistochemical expression of survivin and its correlation with −31G/C gene polymorphism in colorectal cancer

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Abstract

Colorectal cancer (CRC) placed among the most common neoplasm. Survivin is a member of the inhibitor apoptosis gene family. This gene could be associated with aggressive behavior in numerous types of cancers. The aim of the present study was to evaluate the immunohistochemical expression of survivin gene and its correlation with −31G/C polymorphism in CRC patients. This case–control study was performed on 90 cases: 30 adenocarcinoma, 30 adenomatous polyp, and 30 normal colon. Immunohistochemical expression of survivin evaluated on formalin-fixed paraffin-embedded tissue and −31G/C polymorphism was analyzed by polymerase chain reaction-restriction fragment length polymorphism analysis. Results showed that the subjects carrying C/C genotype with 43.3% ($p=0.002$, OR = 12.188, CI = 2.530–58.720) and G/C genotype with 43.3% ($p=0.032$, OR = 4.432, CI = 1.133–17.341) significantly had increased risk of CRC compared with subjects carrying GG genotype. Allelic frequencies showed statistically significant difference ($p=0.001$) among adenocarcinoma (G = 35%, C = 65%), adenomatous (G = 43.3, C = 56.7), and normal group (G = 68.3, C = 31.7). Immunohistological evaluation showed nuclear survivin protein expression in patients with the CC genotype higher than in patient with the GG and GC genotypes ($p=0.002$). The results suggest that C allele of −31G/C polymorphism in survivin might be cooperative in CRC development.

Keywords Survivin · Gene · Polymorphism · Immunohistochemistry · Colorectal cancer

Introduction

Colorectal cancer (CRC) is a disease originates from the epithelial cells lining the large intestine. It is a malignant neoplasm in the alimentary canal with over 1 million new cases in each year [1, 2]. CRC is the third most common type of cancers and the fourth most common cause of cancer-related mortality worldwide [3]. In Iran, CRC is, respectively, the third and fourth most common cancers in men and women [4, 5], and average age of CRC patients is 11.6 for men and 10.5 for women [6].

Although CRC is the most malignancy of digestive system, but the main cause of it has not been clearly understood [7, 8]. Incidence of this neoplasm varies between different populations due to varieties in the genetic background, various racial distribution, nutrition habits, and lifestyle [9–12]. Several researchers have studied on specific biomarkers that could be applied in exact and quick diagnosis of diseases and the early detection of cancers to increase the probability of a better treatment [1, 13].

It believes that CRC could be induced by mutation in tumor suppressor genes and overexpression of inhibitors of apoptosis proteins (IAPs) genes [14, 15]. Survivin is a unique member of the IAPs family. Survivin gene is located on chromosome 17q25 [16]. This gene has five splice variant and it gives rise to five transcripts [17]. Survivin has dual function in apoptosis and cell proliferation. Survivin promoter harbors cell cycle-dependent elements (CDE) and cell cycle gene homologue region (CHR)

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controls at the transcription. Survivin expression occurs with peak at the G2/M phase of the cell cycle and declines rapidly in G1 phase. Thus, it is assumed to play an important role in mitosis regulation and cellular homeostasis [18]. Survivin gene is strongly expressed in embryonic and fetal tissues; studies also detected this protein indifferent adult tissues [19].

Expression of survivin has been shown in different pathological conditions such as malignant tumors and developing cancers. Overexpression of survivin was demonstrated in tumors of colon, stomach, pancreas, liver, breast, and other types of cancers [20]. High level of survivin expression in cancer cells and minimal expression in normal tissues suggested that this gene could play an important role in tumor genesis and survival of tumor cells. However, there is a little information about up-regulated expression of survivin in cancer, and some studies proposed molecular mechanisms of genetic alternation in cancer cells [21].

Several single-nucleotide polymorphisms (SNPs) have been identified within the promoter region of the survivin gene. One of these is located at position −31 within CDE/CHR repressor-binding motif. Survivin −31G/C polymorphism (rs9904341) alters CDE-binding motif and results in mutation, functional disruption, and overexpression of the survivin gene [14]. There are several evidence showing the correlation between increased expression of survivin gene and higher risk of cancer and reduction in survival of cells [22].

Recent studies showed that some of SNPs could have an important role in alteration of biological processes [23]. The previous studies showed that −31G/C (rs9904341) survivin gene polymorphism could modulate some malignancies such as lung, breast, and colorectal cancers. In recent years, studies regarding frequently distributed genotypes of −31G/C SNP in CRC have shown that CC + GC genotype could develop cancer [24].

Results of several studies in CRCs demonstrate the expression rate of survivin in colorectal tissues at a range of 21–63.5% [25–27]. Furthermore, due to different expressions in tumoral tissues and important role of survivin gene in CRCs, this gene probably could be a useful biomarker for determination of treatment strategies in patients with CRC. According to the previous findings regarding survivin expression and distribution of genotypes, it seems that this biomarker has probably a role in the early prognosis and diagnosis in CRC progress and development of malignancy. Since some of the polymorphism and situation of promoter region of the survivin gene may impress the activities of the gene, therefore, in the present study, we aimed to study the association between the survivin −31G/C (rs9904341) SNP and immunohistochemical expression of survivin in CRC patients and normal colon groups.

Methods

This case–control study was conducted on 90 colorectal specimens selected from the pathologic files archive at Ali-ebne-Abitaleb Hospital, Zahedan University of Medical Sciences (ZAUMS), Zahedan, Iran. All samples had been obtained by endoscopic biopsy from patients admitted from 2011 to 2016. The inclusion criteria for the selection of archival histological specimens were suitable formalin-fixed paraffin-embedded tissue from patients with colorectal cancer, precancerous lesion, and normal tissue. The clinical data without the patient's name were obtained from patients' clinical records including age, sex, tumor location, and lymph-node metastasis. The exclusion criteria were the samples from patients with immune disorders, atrophied samples, and specimens with deficits in records of the necessary information. The ethics committee of Zahedan University of Medical Sciences approved the project (No: IR.ZAUMS.REC.1394.53). In the present study, selected specimens were classified according to the histological diagnosis: 30 patients with colorectal carcinoma, 30 patients with adenomatous polyp, and 30 patients with normal colon.

Polymorphism genotyping

To investigate the polymorphism of −31G/C, DNA was extracted from the tissue sections using the kit (PR911683EX6041. Sina colon.CO) according to the manufacturer's instructions, and then, polymorphism was investigated using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. The rs9904341 polymorphism was detected using specific primers by PCR-RFLP method. Primers for PCR amplification were as follows:

5′-CGTTCTTTGAAAGCAGTCGAG-3′ (forward),
5′-TGTAGAGATGCGGTGGTCCT-3′ (reverse).

Amplification was performed in a total volume of 20 µl containing 100 ng of template DNA, 1 µl of each primer, and 10 µl of 2× prime Taq premix (Genet Bio, Korea) and 7 µl double-distilled water (ddH_2O). PCR samples were run as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing for 40 s at 65 °C, and 30 s extension at 72 °C, followed by a final extension step at 72 °C for 5 min. Finally, 10 µl of the product digested by restriction enzyme (Fermentas, Vilnius, Lithuania) and digested products subjected to electrophoresis in 4% agarose gel (Invitrogen, USA) and stained with ethidium bromide (Fig. 1).

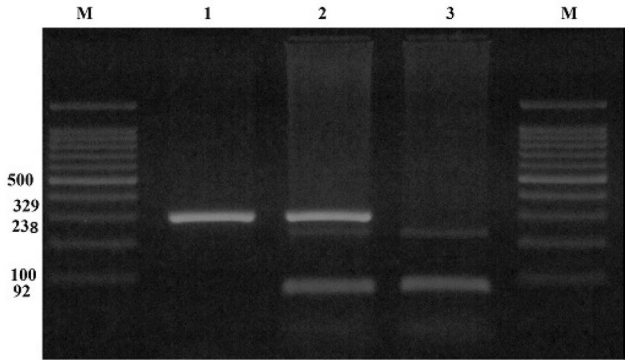

Fig. 1 Electrophoresis pattern of survivin −31G/C (rs9904341) polymorphism. The digestion pattern of ECO01091 restriction enzyme on 4% agarose gel at −31G/C SNP; *M* marker 100 bp, 1 genotype CC (329 bp), 2 genotype G/C (329 + 238 + 92 bp), and 3 genotype G/G (238 + 92 bp)

Immunohistochemical analysis

Immunohistochemical expression was performed on biopsy sections of patient with adenocarcinoma, adenomatous polyp, and normal colorectal tissues using polyclonal anti-survivin antibody (Abcam 469, USA). Time, temperature, and storage condition were according to the manufacturer's instruction. Selected tissue blocks were cut to 4 µm thin sections using fully automated microtome (Lecia, RM2255, Germany) and mounted on HistoGrip (Histogrip50x CL00-8050-Cedarla Co) coated glass slides. The section were deparaffinized in xylene and rehydrated through descending series of ethanol. Then, section for antigen retrieval transferred for 20 min at 120 °C under pressure in sodium citrate buffer (10 mM solution, pH 6) in an autoclave (Medical Prestige, Series 210003 Classic, England). After that, the sections were placed at the room temperature to cool down, and washed with Tris-buffered saline (TBS) (Santa Cruz Biotechnology, Inc., USA). The slides to inhibit endogenous peroxidase activity were incubated in 3% hydrogen peroxide for 10 min and washed with TBS. Blocking protein was applied for 10 min. Then, the sections were incubated over night at 37 °C (for 18 h), with a rabbit polyclonal anti-survivin antibody (Abcam 469, USA) and placed in TBS. The sections were incubated with Biotinylated secondary antibody for 30 min at room temperature and washed with TBS. The slides were incubated in Avidin D-HRP complex and eluted with TBS for 30 min. The sections were incubated with HRP substrate containing diaminobenzidine (DAB) for 20 min and rinsed with distilled water. The section counterstained with Mayer's hematoxylin for 5–8 min and rehydrated. Then, clearing and mounting of sections on glass slides were done. Positive and negative controls were performed at the same time for each section. The positive control was the section sample of colorectal cancer. Negative control was obtained by incubation of slides in TBS and omitting the primary antibodies.

The evaluated immunohistochemistry (IHC) scoring performed by two expert histologists that were blind regard the pathological diagnosis, under a light microscope (Zeiss, Germany) with a 400× magnification. The evaluation nuclear survivin expression according to proportion (extent) and intensity of immune reactivity positive cells; percentage of positive tumor cells was as follows: (0) less than 5% positive cells, (1) between 5 and 25%, (2) between 25 and 50%, (3) between 50 and 75%, and more than 75% tumoral cells. Intensity score was defined as negative (0), weak (1), moderate (2), and strongly positive (3). Survivin-positive nuclear staining was observed in (52.5%) of colorectal samples. Ultimately, final score of sections were calculated by multiplying the extent by immune staining intensity range of 0–12. Scores were classified semi-quantitatively as follows: 0–4 as negative, 5–8 as weak, and 9–12 as strong immune staining [1, 13, 28, 29].

Statistical analysis

Statistical analyses were conducted using the statistical software package SPSS16. The data were reported as mean values ± SD for parametric variables and percentages for non-parametric values. Evaluated odd ratio (OR) and 95% confidence intervals (95% CI) from logistic regression analysis were investigated association between genotypes and CRC. To identify statistical difference between groups, Kruskal–Wallis test used. Also for analyzing the association between survivin and demographic parameters, Pearson's Chi-square test was done.

Results

The mean age of total subjects was 44.3 ± 18.25, in the range of 5–83 years. The majority of CRC cases were female (20; 66.7%), well differentiated (26; 86.7%), mucinous adenocarcinoma type (18; 60%), and located in the sigmoid colon (10; 33.3%). Study subject were divided into three groups: adenomatous, adenocarcinoma, and normal. More details on the clinical characteristics of study patients are listed in (Table 1).

The results showed that, in the specimens with adenocarcinoma, there was a significant association among survivin expression and age ($p < 0.001$) and gender ($p < 0.05$), but there was no significant association among survivin expression and localization, lymph-node metastasis, and type of tumor. According to the results, positive expression of survivin protein in adenocarcinoma, adenomatous, and normal colorectal tissues which were significantly different statistical analysis showed that survivin expression in CRC

Table 1 Association between clinicopathological data and survivin expression in colorectal cancer tissue specimens

Parameter	Adenocarcinoma	*p* value
Age (years)	53.53 ± 16.794	0.001
Sex		0.005
Male	10 (33.3%)	
Female	20 (66.7%)	
Localization		0.329
Appendix	3 (10%)	
Cecum	7 (23.7%)	
Ascending colon	6 (20.0%)	
Transverse colon	0 (0%)	
Descending colon	0 (0%)	
Sigmoid colon	10 (33.3%)	
Ano rectal	4 (13.3%)	
Lymph node		0.144
Present	14 (46.7%)	
Absent	16 (53.3%)	
Differentiation		0.736
Well	26 (86.7%)	
Moderate	3 (10.0%)	
Poorly	1 (3.3%)	
Type		0.231
NOC	9 (30.0%)	
Signet ring cell	3 (10.0%)	
Mucinous	18 (60.0%)	

The data were reported as mean values ± SEM

tissue was significantly higher than adenomatous and normal specimens ($p < 0.001$) (Table 2; Fig. 2).

The results showed that genotype distribution was significantly differently among three groups. The frequency of the genotype was significantly different among adenocarcinoma compare to the other groups (GG:GC:CC of 50%, 36.7%, 13.3% vs. 26.7%, 33.3%, 40% vs. 13.3%, 43.3%, 41.3%; $p < 0.05$). Allelic frequencies showed statistically significant differences among adenocarcinoma (G = 35%, C = 65%), adenomatous (G = 43.3%, C = 56.7%), and normal group (G = 68.3%, C = 31.7%) ($p < 0.001$) (Table 3). Regarding the clinicopathological features, the current study suggested that there were no relationship among distribution of genotype and age, gender, type of tumor, lymph node, and distant metastasis. The result of immunohistochemical evaluations of tissue specimens showed that cancer cell with the G/C or C/C genotype and C allelic had significantly higher nuclear

Table 2 Comparing the expression levels of survivin in tissue samples of adenocarcinoma, adenomatous polyp, and normal colorectal tissues

Group	*N*	Survivin positive (mean ± SEM)	*p* value
Normal	30	3.27 ± 0.87	< 0.001
Adenomatous polyp	30	5.94 ± 0.74*	*F* = 98.986
Adenocarcinoma	30	6.57 ± 1.20$^{\#,¥}$	

*$p < 0.001$, compared with normal

$^{\#}p < 0.001$, compared with normal

$^{¥}p = 0.034$, compared with adenomatous polyp

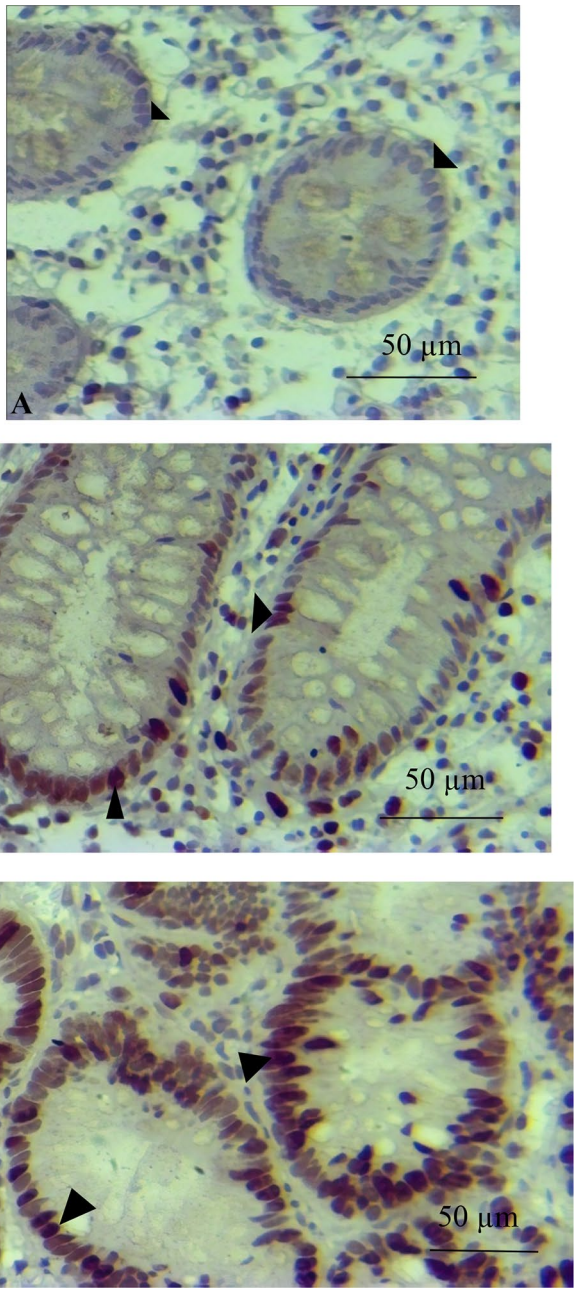

Fig. 2 Expression of survivin in normal (**a**), adenomatous polyp (**b**), and adenocarcinoma (**c**) human colorectal specimens (IHC, ×400 magnification). Survivin-positive expression in cells (black arrow) and healthy cells (black triangle) is shown

Table 3 Distribution of genotype −31G/C polymorphism in the adenocarcinoma, adenomatous, and normal tissue samples

Genotype/alleles	Normal (%)	Adenomatous polyp (%)	*p* value	Odds ratio	Adenocarcinoma (%)	*p* value	Odds ratio
GG	15 (50.0)	8 (26.7)	Ref = 1	–	4 (13.3)	Ref = 1	–
GC	11 (36.7)	10 (33.3)	0.389	1.705 (0.507–5.729)	13 (43.3)	0.032	4.432 (1.133–17.341)
CC	4 (13.3)	12 (40.0)	0.017	5.625 (1.359–23.274)	13 (43.3)	0.002	12.188 (2.530–58.720)
GC + CC	15 (23.8)	22 (34.9)	0.066	2.750 (0.934–8.100)	26 (41.3)	0.004	6.500 (1.820–23.213)
G	41 (68.3)	26 (43.3)	Ref = 1	–	21 (35.0)	Ref = 1	–
C	19 (31.7)	34 (56.7)	0.006	2.822 (1.338–5.950)	39 (65.0)	0.000	4.008 (1.875–8.568)

The frequency of genotypes GG:GC:CC: Pearson's Chi-square = 0.015 < 0.05 (95%)

The frequency of genotypes GG:GC + CC: Pearson's Chi-square = 0.007 < 0.05 (95%)

Allele frequency: Pearson's Chi-square = 0.001 < 0.05 (95%)

Table 4 Association between clinicopathological data and distribution of genotype in −31G/C tissue specimens

Region	−31G/C polymorphism				Asymp. sig. (two-sided)
	GG	GC	CC	Total	
Appendix	1 (33.3%)	2 (66.7%)	0 (0.0%)	3	0.183
Cecum	0 (0.0%)	4 (57.1%)	3 (42.9%)	7	
Ascending colon	2 (33.3%)	2 (33.3%)	2 (33.3%)	6	
Sigmoid colon	1 (10.0%)	5 (50.0%)	4 (40.0%)	10	
Anorectal	0 (0%)	0 (0%)	4 (100%)	4	
Lymph node					0.287
Yes	2 (14.3%)	8 (57.1%)	4 (28.6%)	14	
No	2 (12.5%)	5 (31.3%)	9 (56.3%)	16	
Type					0.116
Non-mucinous	2 (22/2%)	6 (66/7%)	1 (11.1%)	9	
Mucinous	1 (33/3%)	1 (33/3%)	1 (33/3%)	3	
Ring	1 (5/6%)	6 (33/3%)	11 (61/1%)	18	
Differentiation					0.658
Poorly	0	0	1	1	
Moderate	0	1	2	3	
Well	4	12	10	26	

survivin expression than the G/G genotype and G allelic (Table 4).

Discussion

The results of the current study showed that the rs9904341 SNP and presence of the CC or GC genotypes were associated significantly increased the risk of colorectal cancer ($p < 0.05$). Our study revealed that survivin expression in adenocarcinoma was significantly higher than adenomatous and normal colorectal specimens ($p < 0.001$). On the other hand, immunohistochemical evaluation showed that nuclear survivin expression in patients with the CC genotype was higher than in patient with the GG and GC genotypes. In addition, the C allele increased the risk. Studies and strong evidences suggested that C allele could be a risk allele for many malignancies. The present study did not demonstrate significant associations between −31G/C polymorphisms and clinicopathological data. Our finding was agreement with the result reported by TU et al. [30], and their results indicate that the survivin genetic variants are related to EGFR mutation in lung carcinoma patient and might contribute to pathological development to NSCLC. Yazdani et al. examined the association between −31G/C polymorphism and risk of papillary thyroid carcinoma (PTC). Their study showed that the frequency of GC or CC genotypes and C allele in the patient with PTC was significantly higher than control group. In addition, a significant increase in the allele C was found in patients with more aggressive clinical manifestation including lymphatic involvement compared to the control group [31]. Zahedi et al. demonstrated the association between −31G/C polymorphism and the endometrial cancer. They showed that the presence of allele −31C could significantly increase endometrial tissue malignancy compared to the healthy control [32]. Wang et al. revealed a significant association between promoter polymorphism of survivin gene and the risk of urothelial cancer (UC). They showed that the incidence of CC (34.7%) and GC (47.9%) genotypes was significantly higher in patients with UC than control group [33]. On the other hand, Bogdanovic et al. reported that the frequency of GG genotype was 58.7% in patients with UC, and they determined the patient carrying the GG genotype which had a significantly increased risk compared to the patients with GC or CC genotypes [34]. Hossein Pour Feizi et al. have also demonstrated that CC and GC genotype distribution was not statistically significant between breast cancer and control group [35]. Yamak et al. examined association between survivin gene polymorphisms

and risk of colon cancer. They reported that survivin −31G/C polymorphism can induce significantly higher risk of cancer development which is consistent with our study [36]. Bogdanovic et al. stated that patient carrying GG genotype had a significantly overexpression of survivin protein, advance tumor grade (G2), and higher stage (T2–T4) tumors compared with patient carrying the GC or CC genotype [34]. Kawata showed that patients with CC genotype had significantly higher risk for bladder cancer compared to patient with GG and GC genotype. In addition, immunohistochemical evaluation indicated that patient carrying CC genotype had significantly higher survivin expression than patient with GG and GC genotype [37]. Qin-Qin et al. showed that the survivin −31G/C polymorphism was associated with increased cancer risk in overall population as well as Asians. The divergences in the result could be due to the different geographic distribution or ethnic between the study populations. The results have shown that overexpression of the survivin protein play an important role in the adenocarcinoma patient higher than adenomatous and normal patient has. The result showed that there was a significant association between survivin expression and age and gender, but there was no significant association between survivin expression and localization, lymph-node metastasis, and type of tumor. Negi et al. in studies on survivin expression in squamous cell carcinoma (SCC) showed that survivin expression in normal mucosa, leukoplakia, and oral squamous cell carcinoma was 20%, 53.33%, and 80%, respectively, and was significantly different between three groups [38]. Abdelrahman et al. demonstrated a significant association between expression of survivin protein and greater differentiation of the astrocytic glioma. In addition, they found a significant association among survivin index and the grade, age, and tumor size [39]. Shintani et al. examined the IHC expression of survivin in gastrointestinal cancers and reported a higher nuclear expression in patients with colorectal cancer than in control group. In addition, there was no significant association between expression of survivin and clinicopathological parameters. On the other hand, in gastric cancer, the level of survivin expression was associated with age, tumor differentiation, and lymphatic invasion ($p < 0.05$) [20]. Antonacopoulou et al. also revealed higher survivin expression in colorectal carcinoma compared to the normal tissue. In addition, nuclear survivin expression correlated with the primary site of cancer. Level of survivin expression higher in tumors of right colon compared to the left colon or rectum [17].

Gayathri and Rao found significantly stronger survivin expression in oral squamous cell carcinoma (OSS) than in normal mucosa and dysplastic leukoplakia. Regarding histopathological variables, they showed that the expression of survivin was significantly stronger in moderately and poorly differentiated OSS than in well-differentiated cases of OSS [16]. Jakubowska et al. observed a positive expression of survivin in the tumor tissue of 84.2% of patient with CRC. In addition, the nuclear immune reactivity of survivin was found in tumor mass located in the rectum and in patient with distant metastases [3]. Tastekin et al. showed a direct correlation between survivin level in oropharyngeal and survival rate [28].

On the other hand, Li et al. showed that survivin expression in gastric cancer was higher than that of normal tissues. Survivin expression was found in 65% human gastric cancer samples [40]. The finding of above-mentioned studies might be limited by several factors such as different sample size, patient's selection, epidemiological and geographic factors, and genotyping methods in different study.

In conclusion, this study showed strong evidence of the association between expression of survivin protein and weakly prognosis in advance colorectal cancer. On the other hand, the results revealed an association between polymorphism −31G/C and pathological aggressive behavior of colorectal cancer. There is a relationship between −31G/C polymorphism and high nuclear expression of survivin. Therefore, there might be cooperative influence on risk of colorectal cancer. Evidences showed that expression of survivin was changed in cancer. It seems that this protein probably can be a useful biomarker and strong tool to study the biology of cancer, and has the ability to be used in screening, diagnosis of early stage cancer, prognosis, target therapy, and advancement of response to cure in cancer. The findings need to be interpreted with caution and, also, must further examine other polymorphisms in regulatory genes and the association between them in patients with CRC.

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Author contributions Zahra Heidari, Asiyeh Hakimi, and Bita Moudi—study design, histological tissue processing; microscopy and quantification of the sections; data acquisition; illustrations; manuscript writing. Hamidreza Mahmoudzadeh-Sagheb—participated in the study design and literature review. All authors had read the manuscript, and are in agreement with the content of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interests to disclose.

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