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ORIGINAL ARTICLE

# Survivin -31G/C promoter polymorphism and sporadic colorectal cancer

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**Abstract**
*Introduction* Survivin is an apoptotic inhibitor, plays an important role in cell cycle regulation, and may be involved in the development and progression of cancer. A common polymorphism at the *survivin* gene promoter (-31 G/C) has been shown to influence survivin expression and the risk for cancer.
*Aim* The aim of the present study was to investigate whether this polymorphism could be involved in the sporadic colorectal cancer (CRC) development, prognosis, and survival.
*Materials and methods* The -31G/C polymorphism of *survivin* promoter was analyzed by polymerase chain reaction (PCR) restriction fragment length polymorphism method in biopsies from 312 patients with sporadic CRC and 362 healthy individuals. Survivin messenger RNA (mRNA) expression in CRC tissues was detected by quantitative reverse transcriptase PCR.
*Results and discussion* The genotype frequencies for -31GG, -31GC, and -31CC were 21.79%, 41.99%, and 36.22% in CRC patients and 33.98%, 45.03%, and 20.99% in healthy subjects, respectively. The frequencies of the *survivin* -31C allele and CC genotype were significantly higher in CRC patients than in healthy subjects ($p<0.0001$). Homozygotes for the -31CC *survivin* genotype, expressed 1.6-fold higher mRNA levels of *survivin* compared to cases with the -31GG and -31GC genotypes.
*Conclusion* The -31CC genotype of *survivin* promoter is associated with CRC and may be a risk factor for CRC.

**Keywords** Survivin · -31G/C polymorphism · Colorectal cancer

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## Introduction

The inhibitor of apoptosis proteins (IAPs) are a family of anti-apoptotic proteins that inhibit initiator (caspase-9) and effector caspases (caspase-3 and caspase-7) and thus prevent apoptosis [1, 2]. Survivin, a recently found member of the IAPs family [3, 4], is involved in cell cycle regulation and in inhibition of the apoptotic pathways [5, 6]. To date, eight human IAP family members have been determined. These proteins exhibit one to three baculovirus IAP repeat (BIR) domains, which is important for their anti-apoptotic effect and a C-terminal RING motif or caspase

activation recruitment (CARD) domain [7]. Unlike other IAPs, survivin contains only one copy of a BIR and no RING finger motif [3]. Two major apoptosis pathways are known the mitochondrial pathway and the death receptor. *Bcl-2*, which blocks mitochondrial cytochrome c release in the cytosol, has been shown to inhibit rh3, the first of these two pathways [8]. Survivin blocks a common downstream part of both apoptosis pathways, by directly inhibiting terminal effector caspase-3 and caspase-7 and by interfering with caspase-9 activity/processing [8]. Additionally, survivin counteracts apoptotic stimuli induced by interleukin (IL)-3, Fas (CD95), Bax, tumor necrosis factor α, caspases, anticancer drugs, and X-irradiation [9, 10].

One of the most significant features of survivin is its different distribution in cancer compared to normal tissues. Survivin is strongly expressed in embryonic and fetal tissues but is undetectable in most terminally differentiated normal adult tissues [3].Normal tissues that express survivin include thymus, $CD34^{+}$ bone marrow-derived stem cells at low levels, and the basal colonic epithelium [6]. By contrast, dramatic overexpression of survivin compared to normal tissues was demonstrated in tumors of lung, breast, colon, stomach, esophagus, pancreas, bladder, uterus, ovaries, large-cell non-Hodgkin's lymphoma, leukemias, neuroblastoma, melanoma, and non-melanoma skin cancers [6]. In genome-wide searches, survivin constituted the fourth top "transcriptome" in cancers of colon, lung, brain, breast, and melanoma, but its expression was low or undetectable in the normal tissue of the same specimens [11].

Survivin is expressed in a cell-cycle-dependent manner, with a peak in the G2/M phase of the cell cycle, when it is associated with the microtubules of the mitotic spindle, and exhibits a rapid downregulation in the G1 phase [5]. This is controlled at the transcriptional level and mediated by cell-cycle-dependent elements (CDEs) and cell cycle homology regions (CHRs) located in the proximal region of the *survivin* promoter [12]. Several single-nucleotide polymorphisms were identified within the promoter region of the *survivin* gene, one of which is located at the CDE/CHR repressor binding site (-31G/C). This polymorphism has been associated with overexpression of survivin at both messenger RNA (mRNA) and protein levels and aberrant cell-cycle-dependent transcription, mediated through the functional disruption of binding at the CDE/CHR repressor motifs in a number of cancer cell lines [13].

Colorectal cancer (CRC) is one of the most frequent cancers worldwide. The development and progression of CRC involves unregulated epithelial cell proliferation associated with a series of accumulated genetic alterations [14]. There is evidence that prolonged survival of such genetically unstable colorectal epithelial cells, leading eventually to their ultimate malignant transformation, is associated with progressive inhibition of apoptosis. In patients with CRC, expression of survivin has been associated with unfavorable outcome, shortened survival, and reduced tumor cell apoptosis in vivo [15, 16]. However, the -31G/C polymorphism within the CDE/CHR repressor element of the promoter have been studied only in cervical, lung, and gastric cancer to date [17–19], whereas only in lung and gastric cancers an association was found with risk for cancer development.

Therefore, based on these data, our aim was to investigate whether the genetic polymorphism -31G/C located in the CDE/CHR repressor element of the human *survivin* promoter could represent a risk factor for CRC.

## Materials and methods

*Patients* The study population consisted of a well-documented series of 312 patients (158 men and 154 women; mean age, 67.7 years; range, 33–93 years), who underwent surgery for CRC at our institutions between January 2000 and December 2006. Patients with hereditary CRC and inflammatory bowel disease-related cancers were excluded from the study. None of the patients underwent preoperative chemoradiotherapy. The tumor was located in the right colon in 63 cases (20.19%), in the left colon in 162 cases (51.92%), in the transverse colon in 13 cases (4.16%), and in the rectum in 74 cases (23.72%). The histological grade was assessed according to WHO criteria [20]: 40 tumors (12.82%) were well differentiated, 241 (77.24%) moderately differentiated, and 31 (9.93%) poorly differentiated. According to the International Union Against Cancer classification and TNM staging system [21], 46 of the tumors (14.74%) were stage I, 107 (34.29%) stage II, 114 (36.54%) stage III, and 45 (14.42%) stage IV (Table 1). The 362 healthy controls used were randomly selected from a pool of healthy volunteers who visited the hospital during the same period. The study was approved by the hospitals review board, and written informed consent was obtained from each participant.

*Determination of -31G/C polymorphism* DNA was extracted from biopsies of CRC patients and from blood mononuclear cells of healthy controls using the AllPrep DNA/RNA/Protein mini kit of Qiagen. The primers used were 5′-GTTCTTTGAAAGCAGTCGAG-3′ (forward) and 5′-GCCAGTTCTTGAATGTAGAG-3′ (reverse). The amplification was started with an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 90 s, and extension at 72°C for 90 s, and completed with a final elongation at 72°C for 5 min. The 341-bp amplimers were then digested with the restriction enzyme *Eco*O109I (New England

**Table 1** Histopathological characteristics of colorectal cancer samples at diagnosis

| Characteristics | Colorectal cancer patients (*n*) |
|---|---|
| Tumor location | |
| Rectum | 74 |
| Left colon | 162 |
| Right colon | 63 |
| Transverse | 13 |
| Tumor size | |
| ≤4 cm | 162 |
| >4 cm | 150 |
| Growth pattern | |
| Ulcerative | 136 |
| Protruding | 176 |
| Differentiation | |
| Good | 40 |
| Moderate | 241 |
| Poor | 31 |
| TNM stage | |
| I | 46 |
| II | 107 |
| III | 114 |
| IV | 45 |

Biolabs) at 37°C for 18 h. Digestion patterns were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide. The G allele is cleaved by the enzyme, generating two fragments (236 and 105 bp), whereas the C allele is not digested.

*Reverse-transcriptase PCR* Total RNA was extracted from 32 fresh biopsies from the pathologic area of CRC patients using the AllPrep DNA/RNA/Protein mini kit of Qiagen according to the manufacturer's instructions. Hereafter, reverse transcription was performed by incubating 1 μg total RNA for 1 h at 42°C in the presence of 500 μg/mL of Oligo dT 12–18, 10 mM deoxyribonucleotide triphosphates, 5× first-strand buffer, 0.1 M dithiothreitol, and 200 U/ml M-MLV reverse transcriptase (Invitrogen). Assessment of the survivin mRNA levels was performed by employing the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels as a reference gene. The following pair of primers were used: (1) GAPDH (Fw) 5′-CATCTCTGCCC CCTCTGCTG-3′ and (Rv) 5′-CGACGCCTGCTTCAC CACCT-3′ resulting in a 439-bp fragment and (2) survivin (Fw) 5′-TGCTCTTGTTTTGTC TTGAAAGTGGC-3′ and (Rv) 5′-GGAGGCACAGGTGT GACAGATA-3′ resulting in a 332-bp fragment. Quantitation of expression levels was performed on a PhosphorImager using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

*Statistical analysis* Allele frequencies were compared with the chi-square with Yate's correction using S-Plus (v. 6.2, Insightful, Seattle, WA). Odds ratios (ORs) and 95 confidence intervals (CIs) were obtained with GraphPad (v. 3.00, GraphPad Software, San Diego, CA). The *p* values are all two-sided. Strong association (significance) was assumed at $p<0.01$. A weak but still significant association, meriting attention, was considered for values ranging between 0.01 and 0.05.

**Table 2** Frequency distribution of the *survivin* promoter -31G/C polymorphism in patients and controls

| | CRC patients (*n*=312) | Controls (*n*=362) | *p* value; OR (CI 95%) |
|---|---|---|---|
| -31 G/C Genotype | | | |
| GG | 68 (21.79%) | 123 (33.98%) | 1.00 (reference) |
| GC | 131 (41.99%) | 163 (45.03%) | 0.058; 1.45 (0.99–2.11) |
| CC | 113 (36.22%) | 76 (20.99%) | <0.0001; 2.68 (1.77–4.07) |
| Allele frequency | | | |
| G | 267 (42.79%) | 409 (56.49%) | 1.00 (reference) |
| C | 357 (57.21%) | 315 (43.51%) | <0.0001; 1.74 (1.39–2.15) |

## Results

The *survivin* genotype and allele distribution among the 312 CRC patients and 362 healthy controls are presented in Table 2. The observed genotype frequencies were in accordance with Hardy–Weinberg equilibrium ($r=0.963$, $p=0.172$). The -31G/C genotype and allele distribution was significantly different between CRC cases and controls. The -31CC genotype and the -31C allele were overrepresented among the CRC cases ($p<0.0001$).

Concerning the tumor characteristics, no significant association was observed between -31G/C polymorphism and tumor location, tumor size, growth, and differentiation pattern. Interestingly, the -31CC genotype and the -31C allele were found significantly more frequent in stage grouping III and IV than in stage grouping I and II ($p=0.002$ and $p=0.0004$, respectively; Table 3).

**Table 3** Association of the *survivin* genotypes with the tumor stage

| Survivin -31G/C polymorphism | Stage grouping | | *p* value; OR (CI 95%) |
|---|---|---|---|
| | I, II (%) | III, IV (%) | |
| Genotyping | | | |
| GG | 46 | 22 | 1.00 (reference) |
| GC | 58 | 73 | 0.003; 2.63 (1.42–4.86) |
| CC | 49 | 64 | 0.002; 2.73 (1.45–5.13 |
| Allele | | | |
| G | 150 | 117 | 1.00 (reference) |
| C | 156 | 201 | 0.0004; 1.80 (1.31–2.49) |

**Table 4** *Survivin* genotypes in association with CRC overall survival

| Survivin genotype (n) | Overall survival | | | *P* value |
|---|---|---|---|---|
| | Events (*n*) | 5-year survival (%)[a] | Hazard ratio (CI 95%) | |
| GG (68) | 20 | 77.11 | 1.00 (reference) | |
| GC (131) | 27 | 82.44 | 1.26 (0.70–2.39) | 0.41 |
| CC (113) | 45 | 72.50 | 0.59 (0.36–0.98) | 0.042 |

[a] Proportion of survival derived from Kaplan–Meier analysis

Follow-up information regarding survival was available for all the patients (Table 4). The median duration of the follow-up was 36 months (range, 1–60 months). A total of 92 patients suffered cancer-related death during the follow-up period. The hazard ratios of the *survivin* genotypes of the patients for overall specific survival are presented in Table 2. The -31CC genotype was significantly associated with poor survival (Fig. 1). At multivariate analysis, the disease stage and -31CC *survivin* genotype emerge as independent variables of adverse prognostic significance (Table 5).

In order to determine the *survivin* mRNA levels in CRC patients with -31G/C genotypes, quantitative RT-PCR experiments were designed. As illustrated in Fig. 2, in CRC cases, homozygous carriers of -31CC *survivin* genotype expressed approximately 1.6-fold higher mRNA levels of *survivin* than cases with -31GG and -31GC genotypes.

## Discussion

Survivin, a unique anti-apoptotic factor, plays a basic role in cell cycle regulation. Numerous clinical studies have shown that survivin is markedly overexpressed in most common types of cancer, suggesting that transcriptional deregulation is a major mechanism involved in aberrant expression of survivin in cancers [6].

In the present study, we investigated whether the -31G/C polymorphism in the *survivin* gene contribute to the development of CRC and influence the survivin expression levels. In keeping with previous studies that indicate the elevated frequency of -31C allele in lung and gastric cancer patients [18, 19], our results documented that -31CC genotype and -31C allele were associated with a significantly increased risk for CRC. Despite the existing but still rather limited documentation of the *survivin* promoter polymorphism -31G/C with cancer susceptibility, one should point that this is not universally observed between cancers of different origin. Therefore, although the aforementioned evidence in regards to lung and gastric cancer and, as presented in the current series of patients, to CRC, as well, a similar correlation has not been demonstrated in cervical cancer [17–19]. Even though the *survivin* is a well-accepted tumor marker when overexpressed in human cancers, the differential role of its polymorphism remains

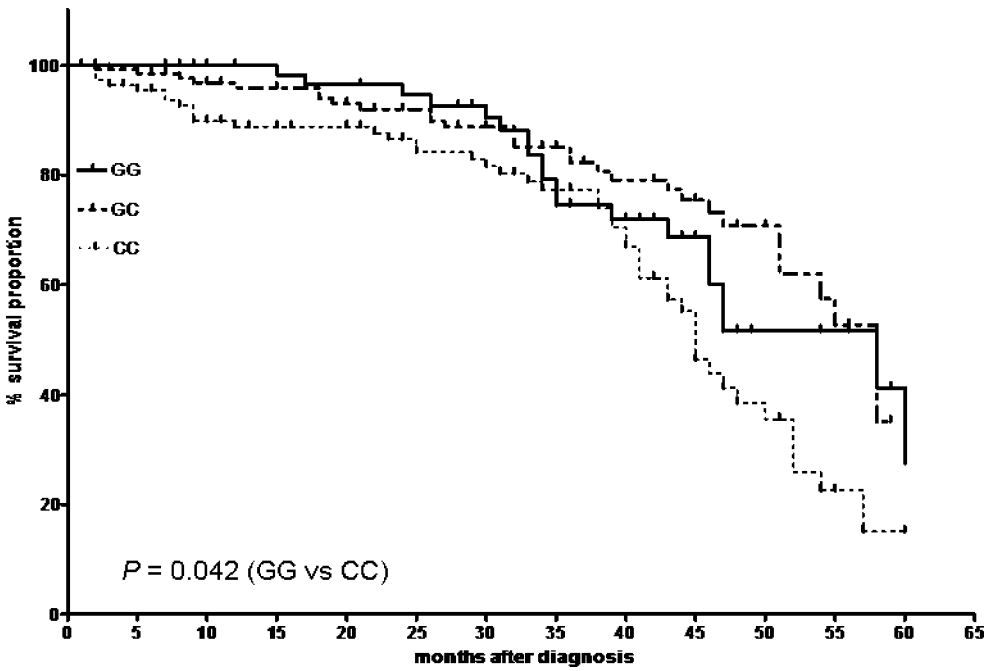


**Fig. 1** Kaplan–Meier survival curves for CRC patients stratified by *survivin* -31G/C genotypes (GG, GC, and CC)

**Table 5** Cox proportional hazard estimation of overall survival

| Covariant | *B* coefficient | SE | *p* value | Correlation coefficient (*r*) | 95% CI for relative risk |
|---|---|---|---|---|---|
| Overall survival | | | | | |
| Disease stage | 0.415 | 0.033 | 0.023 | 0.128 | 0.02–0.264 |
| -31CC *survivin* genotype | 0.321 | 0.031 | 0.027 | 0.124 | 0.01–0.235 |

The analysis for disease stage was performed by grouping patients in two groups (TNM stage I+II and III+IV)

under-explained. A rational explanation for this tumor-dependent difference in risk conferred by the examined *survivin* polymorphism may be attributable to differences in the pathways of carcinogenesis among the various types of human cancers. Furthermore, we observed a strong association between -31CC genotype and advance stage (III and IV) of disease as well as an adverse effect of -31CC carriage at CRC patient's survival. Different stages of disease may reflect altering genotypic and phenotypic tumor profile, at which various polymorphisms, and among them the *survivin* promoter polymorphism, may exert a different but viable role. Disease progression may be more rapid, or the tumor biology might have been determined as aggressive from the early steps of carcinogenesis in -31 CC carriers.

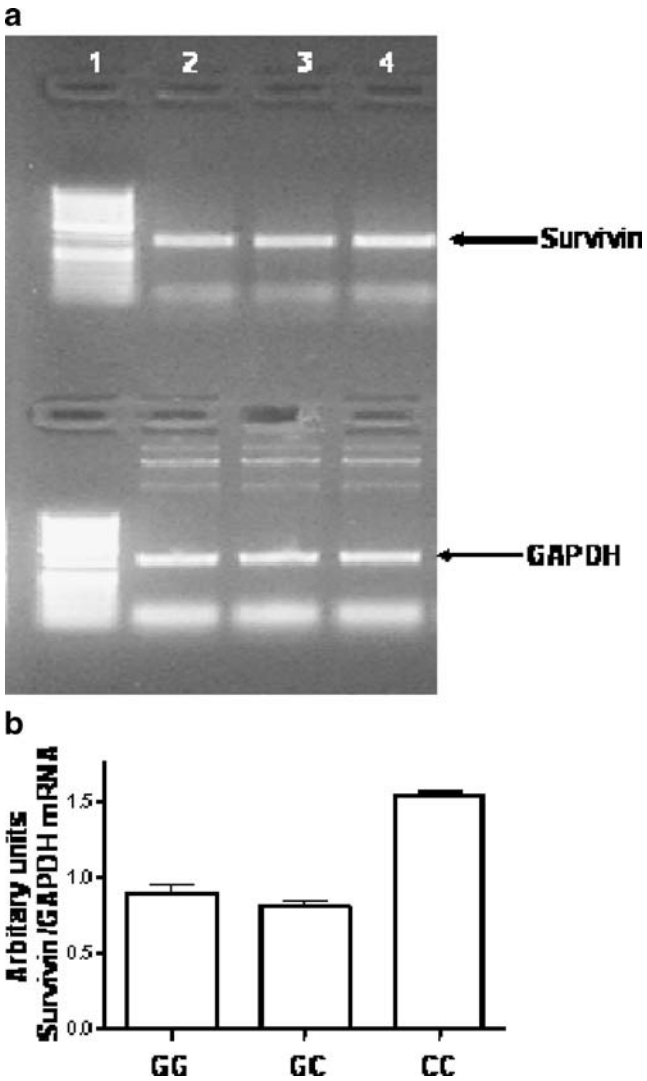


**Fig. 2** **a** Representative RT-PCR analysis. Survivin mRNA expression in biopsy specimens of CRC patients with -31G/C genotypes: GG (*line 2*), GC (*line 3*), CC (*line 4*). *Line 1* represents a molecular weight DNA Ladder (50 bp Fermentas). Normalization for the amount of template was performed using primers specific for the GAPDH gene. **b** Expression levels of Survivin/GAPDH mRNA derived from CRC patients with genotypes GG, GC, and CC utilizing arbitrary units. The results shown represent the average of at least three independent experiments

Recently, using in vitro promoter assay, Jang et al. [18] found that the -31G allele had a significantly lower transcriptional activity than the -31C allele, suggesting that the -31G/C polymorphism influences survivin expression, thus contributing to the genetic susceptibility to lung cancer. Additionally, Xu et al. [13] reported that the presence of the -31G/C polymorphism was more frequent in cancer cell lines and correlated in these cell lines with increased survivin expression at the both mRNA and protein levels. However, Chen et al. [19] found that survivin mRNA was overexpressed in gastric cancer cases but with no significant difference in gastric cancer tissues subgrouped by -31G/C genotypes. Taken these observations in consideration, the association between the -31G/C polymorphism and survivin mRNA expression levels was also tested in our cases. In the present study, we observed that the samples homozygous for -31CC genotype in cancer cases exhibited approximately 1.6-fold higher mRNA levels than the carriers of the -31GG and -31GC genotypes. This finding confirms previous studies [13, 18] but not the recent study by Cheng et al. [19]. The mechanism underlying the overexpression of survivin in cases with -31CC genotype is not known. We can speculate that because the -31G/C polymorphism is located at binding sites for the CDE/CHR repressor in the *survivin* promoter, this polymorphism may influence *survivin* transcription by modifying the banding motif of the CDE/CHR repressor.

Collectively, in the present study, we tested the hypothesis that -31G/C polymorphism in the *survivin* gene can affect the risk of CRC. We found that the -31CC genotype was associated with increased risk for CRC and could be a useful marker for prognosis in CRC. From a clinical perspective, genetic variants and expression patterns of *survivin* may be integrated in potential genetic fingerprint-

ing of patients, linking individual patterns to prediction of clinical outcome and, possibly, to therapy response. The correlation of *survivin* polymorphism with adverse clinical outcome may impose a significant role of the genetic alteration itself and that, by its own, may characterize an aggressive tumor that requires more intense therapeutic intervention. Moreover, in the era of targeted anti-cancer treatments, since the *survivin* is overexpressed in tumor cells and is required for their viability, similar data concerning the genotype and expression of *survivin* may make survivin an attractive theurapeutic target in cancer.

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