Survivin as a Radioresistance Factor, and Prognostic and Therapeutic Target for Radiotherapy in Rectal Cancer

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Abstract

Apoptosis levels have been shown to predict tumor response to preoperative radiochemotherapy in rectal cancer. Recently, the prominent role of survivin, a structurally unique member of the inhibitor of apoptosis protein family, has been shown in colorectal cancer tumorigenesis and prognosis. In this study, we investigated whether survivin plays a direct role in mediating radiation resistance. We used short interfering RNA molecules to decrease survivin in radioresistant SW480 and intermediate radioresistant HCT-15 colorectal cancer cells. This resulted in a significant decrease of survivin mRNA and protein expression with a maximum at 24 to 48 hours after transfection. If irradiated during this sensitive period, an increased percentage of apoptotic cells and an increased caspase 3/7 activity in parallel with a decreased cell viability and a reduced clonogenic survival was shown. These effects were more pronounced in the radioresistant SW480 cell line with a radiation-induced cytotoxicity enhancement factor at 10% and 50% survival of 1.8 to 2.2 for SW480 and 1.5 to 1.7 for HCT-15, respectively. Furthermore, transfection with survivin short interfering RNA increased levels of G2-M arrest and levels of DNA double-strand breaks in irradiated cells. These observations indicate that cell cycle and DNA repair mechanisms may be associated with apoptosis induction in tumor cells that are otherwise resistant to killing by radiation. In a translational study of 59 patients with rectal cancer treated with a combination of radiotherapy and chemotherapy, increased survivin expression was inversely related to the levels of apoptosis, and was also associated with a significantly higher risk of a local tumor recurrence. (Cancer Res 2005; 65(11): 4881-7)

Introduction

Surgery is the predominant therapy for rectal cancer. During the past decades, radiotherapy has increasingly been used together with surgery in order to lower local treatment failures. These treatment protocols have been found to be effective in the treatment of primarily unresectable cancers and to preserve the sphincter in low-lying tumor therapy (1, 2). Although radiation therapy with or without chemotherapy has now become an integral part in the standard treatment of rectal cancer, the current trend is toward refinement of each form of therapy—that is, refinement of surgical techniques and optimization of the therapeutic ratios of radiotherapy and chemotherapy (3).

In general, adenocarcinomas including colorectal cancer tend to be less radiosensitive. Recent data from preoperative radiochemotherapy in rectal cancer, however, indicate a broad variety in tumor response. In our recently completed randomized trial comparing preoperative with standard postoperative radiochemotherapy in rectal cancer (CAO/ARO/AIO-94), a standardized histopathologic tumor regression grading was prospectively applied. Out of 385 patients, 10% revealed complete response, whereas 53%, 14%, 15%, and 8% of patients showed good, moderate, minimal, or no regression treatment response, respectively (2). Despite of uniform treatment protocol, the observed heterogeneity in treatment response is most probably caused by differences not only in tumor size and differentiation but also in the genetic profile of the individual lesions.

In the clinical setting, we and others have previously shown that a high level of pretreatment apoptosis emerged as a significant predictor of tumor regression after preoperative radiochemotherapy in rectal cancer (4–6). In vitro studies, using colorectal cancer cell lines with different intrinsic radiosensitivities further confirmed that spontaneous and radiation-induced apoptosis correlated closely with radiation response (7). Although it is clear from the literature that apoptosis propensity may be regulated by various factors, including p53 and the bcl-2 family, a prominent role of survivin, a structurally unique member of the inhibitor of apoptosis family (8–10), has recently been shown for colorectal cancer tumorigenesis and prognosis (11–16). In a panel of colorectal cell lines, we have previously shown an inverse relationship between levels of survivin expression, rates of spontaneous and radiation-induced apoptosis, and radiosensitivity as determined by the clonogenic assay (7).

In the present study, we sought to assess the effects of attenuation of survivin expression, accomplished through short interfering RNA (siRNA)–induced down-regulation of survivin mRNA and protein, on the response of colorectal cell lines with diverse intrinsic radiosensitivities and different levels of survivin expression (SW480, HCT-15), and to elucidate possible underlying mechanisms by which survivin may mediate radiation resistance. Furthermore, to confirm the observed preclinical data at the clinical level, we analyzed the impact of survivin expression, as determined in the pretreatment tumor material of 59 uniformly treated rectal cancer patients, on the efficacy of preoperative radiochemotherapy.

Materials and Methods

Cell culture. The human colorectal adenocarcinoma cell lines, SW480 and HCT-15, were obtained from the American Type Culture Collection (LGC-Promochem, Wiesbaden, Germany). The cells were maintained in DMEM (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated FCS, 1% sodium pyruvate, and 2 mmol/L glutamine.
(all supplements from Biochrom) at 37°C, 5% CO₂, and 95% humidity. As shown in previous experiments, the cell line SW480 expressed higher amounts of survivin mRNA and protein as compared with the HCT-15 line, it showed lower rates of spontaneous and radiation-induced apoptosis, and was more radioresistant as assessed by the clonogenic assay (7).

Transfection of short interfering RNA and irradiation. The sequence of the double-stranded siRNA (Eurogentec, Scearting, Belgium) used for attenuation of survivin expression corresponded to a 21 bp sequence kindly provided by Dr. Matthias Truss, Pediatric Clinic, University of Berlin (sense, 5'-GUC-GAC-AGA-GAA-AGA-ATT-3'; antisense, 5'-UGG-CUC-UUU-CUC-UUC-CTT-3'). Cells were treated in parallel with a green fluorescence protein (GFP)-siRNA (sense, 5'-GGU-GUG-CUG-UUU-GGA-GGU-CTT-3'; antisense, 5'-GAA-CUC-CAA-ACA-GCA-CAC-CTT-3') as a nonspecific control (MWG, Ebersberg, Germany). Cells (5 x 10⁵) were seeded in 25 cm² flasks 24 hours before transfection resulting in a confluence of the cell monolayer of 50% to 60%. Cells were then cultured in serum-free OptiMEM (Invitrogen, Karlsruhe, Germany) for 4 hours. Survivin-specific siRNA or GFP-siRNA (each 50 nmol) were mixed with OligofectAMINE plus (Invitrogen) and added to the cells. After 4 hours at 37°C, cells were cultivated in DMEM supplemented with 20% heat-inactivated FCS. Twenty-four hours after transfection, cells were irradiated at room temperature using X-ray orthovoltage-irradiation (Stabilipan, Siemens, München, Germany) at 250 kV/15 mA/40 cm focus-surface distance at a dose rate of 1.15 Gy/minute with single doses of 0, 1, 2, 4, 6, and 8 Gy.

Quantitative survivin TaqMan PCR. Total mRNA from the cell lines was isolated using the RNeasy kit (Qiagen, Hilden, Germany). Survivin mRNA subsequences (77 bp), specific for all three known survivin transcripts, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts, mastermixes containing the primer, and the TaqMan probes. The probes were either 5'-labeled with the fluorescent dye 6-carboxyfluorescein for detection of survivin or VIC for GAPDH, and the common 3'-fluorescent quencher 6-carboxytetramethylrhodamine. The remaining free positions were loaded with the required number of sample tubes containing just the respective TaqMan oligonucleotide sets. Aliquots of reaction premixes containing PCR buffer supplemented with the dye 6-carboxy-tetramethylrhodamine, deoxynucleotide triphosphates, and 1.25 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA) were added to each reaction tube by using a BIOMEK 2000 laboratory workstation (Beckman Instruments, Fullerton, CA). PCR amplification and detection was done with an ABI Prism 7000 sequence detection system (Applied Biosystems). Target cDNA amounts were calculated from the simultaneously processed reference DNA strips. Survivin data were correlated to GAPDH cDNA and cDNA amounts were calculated from the simultaneously processed ABI Prism 7000 sequence detection system (Applied Biosystems). Target cDNA amounts were calculated from the simultaneously processed reference DNA strips. Survivin data were correlated to GAPDH cDNA and cDNA amounts were calculated from the simultaneously processed ABI Prism 7000 sequence detection system (Applied Biosystems). Target cDNA amounts were calculated from the simultaneously processed reference DNA strips. Survivin data were correlated to GAPDH cDNA and cDNA amounts were calculated from the simultaneously processed ABI Prism 7000 sequence detection system (Applied Biosystems). Target cDNA amounts were calculated from the simultaneously processed reference DNA strips. Survivin data were correlated to GAPDH cDNA and cDNA amounts were calculated from the simultaneously processed ABI Prism 7000 sequence detection system (Applied Biosystems).

Western immunoblotting. For immunoblotting, cells were washed and lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% deoxycholate] supplemented with protease inhibitors (1 mmol/L phenylmethylsulfonylfluoride, 10 μg/mL pepstatin, 10 μg/mL aprotinin, and 5 μg/mL leupeptin; all from Sigma, Deisenhofen, Germany). Protein concentrations were determined using the bichinchoninic acid protein assay (Pierce, Rockford, IL). Equal amounts of protein (10 μg) were separated on a 12.5% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond C, Amersham, Freiburg, Germany). Membranes were blocked in 5% nonfat dry milk in PBS for 30 minutes at room temperature and probed with rabbit antisurvivin antibodies (dilution, 1:1,000, R&D Systems, Wiesbaden, Germany) overnight at 4°C. Next, membranes were incubated with horseradish peroxidase-linked secondary antibodies (1:200, Dako, Hamburg, Germany) and developed by an enhanced chemiluminescence detection system (ECL, Amersham) and autoradiography ( Biomax film, Kodak, Rochester, NY). To confirm equal protein loading, membranes were subsequently reprobed with a 1:2,000 dilution of an anti-β-tubulin antibody (Biozol, Eching, Germany). For densitometric analysis, scanned autoradiographs were quantified using the AIDA software package (Raytest, Straubenhardt, Germany).

Quantification of apoptosis and caspase 3/7 assay. Apoptotic cells were quantified using the terminal-deoxynucleotidyl-transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) technique (in situ cell death detection kit, Boehringer Mannheim, Germany) according to the manufacturer's instructions on a minimum of 1,000 cells fixed by methanol acetate (3:1). Caspase 3/7 activity was measured in a 96-well microplate format using the Apo-ONE assay (Promega, Mannheim, Germany). Cells (1 x 10⁵) were incubated for 90 minutes in a luciferase substrate mix and luminescence activity was measured in a luminometer (Berthold, Bad Wildbad, Germany).

3-(4,5-Methylthio-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells transfected 24 hours previously with either survivin-specific siRNA or GFP-siRNA were seeded at a density of 2 to 10 x 10⁵ cells/200 μL in a 96-well microplate, grown for 6 hours and subsequently exposed to 8 Gy irradiation. After 24, 48, and 72 hours of incubation at 37°C, 3-(4,5-methylthio-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added (20 μL/well of a 5 mg/mL solution in PBS) for 4 hours. Solubilization of the converted purple formazan dye was accomplished by adding 50 μL/well of 0.01 N HCl/20% SDS and incubating overnight at 37°C. The reaction product was quantified by measuring the absorbance at 570 nm using an ELISA reader (HTS 7000, Perkin-Elmer, Rodgau, Germany) and SoftMax-IT Soft (Perkin-Elmer). All samples were assayed in triplicate.

Cell cycle analysis. Both adherent and detached SW480 and HCT-15 cells (1 x 10⁶/mL) were collected by trypsinization and washed with PBS for 10 minutes by centrifugation at 120 x g. Cells were resuspended in a staining solution containing 1 μg/mL propidium iodide, 4 mmol/L sodium citrate, 1 mg/mL RNase A (Boehringer Mannheim), and 0.1% Triton X-100. Fluorescence-activated cell sorting analysis was done with a Coulter EPICS flow cytometer XL (Coulter, Hialeah, FL) and data were analyzed using the SystemXL-II software (Coulter). At least three independent experiments, each in duplicate, were done for each set of data.

Flow cytometry for phospho-histone γH2AX. For the analysis of Ser^152-phosphorylated histone H2AX, cells were irradiated with 2 Gy, detached by accutase (PAA, Pasching, Austria) treatment for 15 minutes, pelleted by centrifugation (5 minutes, 120 x g) and fixed in 70% ethanol for 2 hours at −20°C. Before antibody labeling, samples were rehydrated with TBS (pH 7.4), centrifugated and incubated in Tris-buffer, 0.1% Triton X-100 for 10 minutes on ice. Next cells were incubated either with an FITC-conjugated anti-γH2AX-antibody at a 1:2,50 dilution or an isotype control antibody (both Upstate, Lake Placid, NY) for 2 hours in the dark. Cells were fixed in 1% paraformaldehyde in PBS and analyzed using a Coulter EPICS-flow cytometer XL (Coulter). The mean fluorescence of the isotype-control was subtracted to eliminate unspecific background staining for every sample.

Clonogenic survival assay. The clonogenic assay was done on single cell suspension as described previously (7). Following transfection with siRNA, cells were plated in complete DMEM into culture dishes and irradiated as described. After 10 to 14 days, colonies were stained with methylene blue solution for 30 minutes and counted using an automatic colony analyzing machine (ACAM, Erlangen, Germany). Next, calculation of survival fractions (SF) was done using the equation SF = colonies counted / cells seeded x (PE / 100), taking into consideration the individual plating efficiency (PE). Survival variables α and β were fitted according to the linear quadratic equation (SF = exp [−α x D − β x D²]) with D = dose using TechPlot-software (TechPlot, Braunschweig, Germany). All experiments were repeated at least thrice. Radiation-induced cytotoxicity enhancement factors at 50% and 10% survival were calculated by transforming the abovementioned equation using x and β values of the individual survival curves.

Preoperative radiochemotherapy protocol. A cohort of 59 patients with locally advanced rectal cancer (International Union Against Cancer stage II and III) received preoperative radiochemotherapy within a prospective protocol (CAO/ARO/AIO-94). All patients received a total dose of 60 Gy in 30 fractions delivered with a linear accelerator and concomitant 5-FU/LV 500 mg/m² for 5 weeks with a 2-week rest period between the first and the second part of radiochemotherapy. Histologic complete response was observed in 56 patients (94%). Two patients developed local recurrences within the irradiated field (1). A follow-up of 7 years has shown a progression-free survival of 70% in patients who achieved a histologic complete response. This radiochemotherapy regimen is currently planned to be applied in a randomized phase III trial (CAO/ARO/AIO 0401).
of 50.4 Gy with daily fractions of 1.8 Gy on 5 consecutive days per week. During the 1st and 5th week of radiotherapy, 5-fluorouracil was delivered concomitantly at a dose of 1,000 mg/m²/day as a 120-hour continuous infusion. Six weeks after completion of preoperative radiochemotherapy, patients were scheduled to undergo surgery. The median follow-up time for these patients was 78.5 months (range, 4.5–120).

**Immunohistochemical staining for survivin and quantification of apoptosis on pretreatment biopsies.** Immunohistochemical staining for survivin was carried out as previously described (17). Before labeling, deparaffinized tissue sections were overlaid with 10 mmol/L citrate buffer (pH 6.0) and heated for 20 minutes in a pressure cooker. Next, slides were blocked with 5% bovine serum albumin in PBS and primary antibodies (ab469, Abcam, Ltd., Cambridge, United Kingdom) were applied at a 1:50 dilution, followed by an incubation with biotinylated anti-rabbit secondary antibody (Dianova, Hamburg, Germany, at a dilution of 1:50 for 1 hour room temperature) and streptavidin/biotinylated alkaline phosphatase for 30 minutes. Finally Fast-Red solution was used as chromogen and hematoxylin (37%) for counterstaining. Negative control slides in the absence of primary antibodies were included for each staining. The mean percentage of positive tumor cells was determined using an Image System (Optimas 6.2, Stemmer PC Systeme, Puchheim, Germany) and assigned to one of the following categories: 0 (<5%), 1 (5–25%), 2 (25–50%), 3 (50–75%), and 4 (>75%). The intensity of survivin immunostaining was scored as: 1+ (weak), 2+ (moderate), and 3+ (intense). The percentage of positive tumor cells and staining intensity were then multiplied to produce a weighted score for each case ranging from 0 to 12. Because of the limited number of patients and to facilitate further statistical analysis, the weighted survivin score was arbitrarily dichotomized: “low-survivin expression” was classified as a survivin score of 5 or below and “high-survivin expression” as a score of 6 or above.

Apoptic cancer cells were quantitated using the TUNEL technique (in situ cell death detection kit, Boehringer Mannheim) as described in ref. (5). The apoptotic index was obtained by dividing the number of apoptotic tumor cells by the total number of tumor cells, multiplied by 100. A minimum of 1,000 cells were counted in 10 random regions of the tumor biopsies using a fluorescence microscope (Axioskop, Leitz, Wetzlar, Germany) equipped with a CCD camera and an Image System (Optimas 6.2, Stemmer PC Systeme).

**Statistical evaluation.** Experimental in vitro data are presented as mean ± SD from three or more independent experiments. Levels of significance for these data were calculated using Student’s t test (Excel, Microsoft, Germany). The association of the dichotomized survivin expression with the mean apoptotic index in the pretreatment biopsies of rectal cancer patients was tested using the Mann-Whitney U test for two independent samples. Local failure rates were calculated using the method of Kaplan-Meier, and differences were analyzed with the log-rank test. The level of significance was 0.05 (two-sided) in all statistical testing.

**Results**

**Down-regulation of survivin mRNA and protein in colorectal cancer cells following transient transfection with survivin-specific siRNA (50 nmol).** Total RNA was extracted at the indicated times after transfection and survivin mRNA was quantitated by a TaqMan PCR. Shown are the relative mRNA levels of survivin in reference to GAPDH expression. Columns, mean; bars, ± SD; *, P < 0.001 versus nontreated control. **A**, Western immunoblots from total cellular proteins extracted 24, 48, and 72 hours after transfection using antibodies against survivin and β-tubulin as a loading control. Data represent one out of three separate experiments. Compared with nontransfected or GFP-siRNA transfected control cells (Fig. 1B), Densitometric analysis yielded an 80% reduction of survivin protein expression at 24 and 48 hours followed by a slight increase at 72 hours.

**Treatment of colorectal cancer cells by short interfering RNA specific for survivin resulted in increased apoptosis and caspase 3/7 activity.** To analyze whether survivin inhibition by siRNA functionally affects spontaneous and radiation-induced apoptosis, we compared the extent of TUNEL-positive cells as well as the caspase 3/7 activity. In non- or GFP-control siRNA–transfected cells irradiation with 8 Gy induced a 2- to 5-fold increase of TUNEL-positive SW480 and HCT-15 colorectal cancer cells at 24 and 48 hours post-irradiation as compared with nonirradiated controls (Fig. 2A). The spontaneous and radiation-induced TUNEL-positive cells were significantly higher (P < 0.02) in survivin siRNA–treated cells at 24 and 48 hours as indicated by a 1.8- to 2-fold increase as compared with nontreated or GFP-siRNA treated cells. As shown in Fig. 2B, survivin siRNA treatment also resulted in a significant increase (P = 0.01) of caspase 3/7 activity in nonirradiated and, more pronounced, in irradiated SW480 and HCT-15 cells as compared with nontreated or GFP-siRNA treated controls.

**Cell proliferation and cell cycle analysis after short interfering RNA treatment.** To determine whether inhibition of
survivin affects cell viability and proliferation in irradiated SW480 and HCT-15, metabolic activity at 24, 48, and 72 hours after irradiation was determined by the MTT assay. Treatment with survivin siRNA resulted in a significant reduction ($P < 0.01$) of the cell viability in nonirradiated SW480 and HCT-15 cells, as well as 24, 48, and 72 hours after irradiation with 8 Gy as compared with GFP- or mock-treated controls (Fig. 3A). Cell cycle analyses done 24 hours after siRNA transfection (i.e., the time of irradiation in the other sets of experiments) revealed an increase in the G2-M fraction in survivin siRNA–treated SW480 and HCT-15 cells, indicating that a larger fraction of both cell lines were blocked in a more radiosensitive stage as compared with nontreated or GFP-control treated cells (Fig. 3B).

Treatment by survivin short interfering RNA resulted in an increased phospho-histone $\gamma$H2AX induced by radiation. To analyze whether inhibition of survivin may also affect radioresistance

**Figure 2.** Survivin attenuation by siRNA treatment affects spontaneous and radiation-induced apoptosis and caspase 3/7 activity. A, the apoptotic cells were determined by TUNEL-staining in nonirradiated SW480 and HCT-15 colorectal cells as well as 48 hours after irradiation with 8 Gy. Columns, mean; bars, $\pm$ SD; *, $P < 0.02$ versus nontreated control. B, caspase activity was determined by a luciferase-based Apo-Glo assay in nonirradiated and irradiated (8 Gy) SW480 and HCT-15 cells. Columns, mean; bars, $\pm$ SD; *, $P < 0.01$ versus nontreated control.

**Figure 3.** Survivin attenuation by siRNA treatment affects cell viability and cell cycle distribution. A, metabolic activity as determined by a MTT assay after transfection of survivin-specific siRNA, GFP-control, or mock-treated cells without (0 Gy) and 24, 48, and 72 hours after irradiation with 8 Gy. Columns, mean; bars, $\pm$ SD; *, $P < 0.01$; **, $P < 0.001$ versus nontreated control. B, 24 hours after transfection, fixed SW480 and HCT-15 carcinoma cells were labeled with propidium iodide and flow cytometry was used to measure DNA content. Data are displayed as one representative out of three experiments. Percentage of cells in the G2-M phase are depicted in the individual plots.
by interaction with repair mechanisms, initial H2AX Ser\textsuperscript{139} phosphorylation was analyzed. As shown in Fig. 4, induction of phospho-histone γH2AX, a marker of recognized DNA double-strand breaks, was increased after survivin siRNA treatment in SW480 cells 20 to 40 minutes after irradiation and remained elevated as compared with GFP- or mock-treated cells.

Survivin as a target for radiation sensitization. To further establish the radiosensitizing ability of survivin siRNA, as observed in terms of induction of apoptosis, cell cycle redistribution, and impairment in DNA double-strand break repair, clonogenic survival assay has been done after treatment with survivin-siRNA in combination with radiation. The clonogenic survival after irradiation with 1 to 8 Gy of SW480 and HCT-15, either treated with survivin- or GFP-control siRNA, was compared with non-siRNA treated cells. As displayed in Fig. 5, inhibition of survivin shifted the survival curves down for both SW480 and HCT-15 cells with a reduction in the shoulder. In both cell lines, 50% and 10% survival were significantly reduced (\(P < 0.03\)) in survivin siRNA–treated cells, resulting in a calculated radiation-induced cytotoxicity enhancement factor of 1.8 to 2.2 and 1.5 to 1.75 for SW480 and HCT-15, respectively (Table 1).

Survivin expression in pretreatment tumor biopsies of rectal cancer patients and correlation with spontaneous apoptosis. Survivin immunoreactivity in 59 tumor biopsies of rectal cancer patients treated within a prospective protocol of preoperative radiochemotherapy was determined and correlated with the levels of spontaneous apoptosis in the same biopsy specimen. The mean apoptotic index for the 21 tumors with high survivin expression was 1.3% (SD ± 0.4%), which was significantly lower (\(P < 0.0001\)) than 2.0% (SD ± 0.6%) observed for the 38 tumors with low-survivin expression.

Survivin expression as a predictor for treatment response to preoperative radiochemotherapy. With a median follow-up of 78 months after preoperative radiochemotherapy and curative resection, the 5-year cumulative incidence of local relapse for the 59 patients was 14%. As shown in Fig. 6, a high-survivin expression was significantly related (\(P = 0.05\)) to an increased risk of local relapse as compared with tumors showing low-survivin expression (6% versus 26% at 5 years).

Discussion

In colorectal cancer, the immunohistochemically determined expression of survivin protein correlated with decreased apoptosis, increased proliferation, increased angiogenesis, and, consequently, unfavorable prognosis (11, 13–18). Microarray-based gene profiling studies recently identified survivin as a "risk-associated" gene signature for unfavorable outcome in this disease (19, 20).

Given such strong evidence implicating survivin’s role in enhancing the malignant potential of colorectal cancer, in this study, we investigated whether survivin also plays a direct role in mediating radiation resistance. We showed that attenuation of survivin mRNA (Fig. 1A) and protein (Fig. 1B) expression by siRNA treatment resulted in an increased rate of spontaneous and radiation-induced apoptosis (Fig. 2A) as well as an increased caspase 3/7 activity (Fig. 2B). Transfection with survivin siRNA decreased cell viability (Fig. 3A), and induced a G2-M arrest (Fig. 3B). Moreover, it also increased the DNA double-strand breaks induced by radiation (Fig. 4), and finally resulted in an increased radiosensitivity as determined in the clonogenic assay (Fig. 5). These effects were more pronounced in the radioresistant SW480
distribution, resulting in an increased G2-M fraction 24 hours after observed that survivin siRNA treatment altered the cell cycle However, there also seemed to be additional mechanisms. First, we related cell death, probably via direct inhibition of caspases (21).

The possible underlying mechanisms by which survivin may enhance cell survival upon radiation exposure remain to be elucidated. The conventional notion is that survivin enhances survival of tumor cells primarily through suppression of apoptosis-related cell death, probably via direct inhibition of caspases (21). However, there also seemed to be additional mechanisms. First, we observed that survivin siRNA treatment altered the cell cycle distribution, resulting in an increased G2-M phase fraction 24 hours after transfection (Fig. 3A). Thus, at the time of irradiation, the cells were blocked in a more radiosensitive stage of the cell cycle. Kappler et al. (22) have also recently shown a marked G2-M arrest, an impaired mitosis, and an increased number of polyploid cells after survivin-specific siRNA treatment in five human sarcoma cell lines. This is in line with our observations of a reduced cell viability in the MTT test (Fig. 3A) and a reduced plating efficiency in the clonogenic assays after survivin siRNA treatment alone (Table 1). Furthermore, we found a higher incidence of DNA double-strand breaks after irradiation, as indicated by a higher amount of Ser15-phorylated histone γH2AX staining 20 to 360 minutes after survivin siRNA treatment (Fig. 4; ref. 23). Using adenoviral vectors containing a dominant-negative survivin construct, Chakravarti et al. (24) also reported a decreased DNA repair capacity of glioblastoma cells upon radiation exposure as measured by a comet assay. Thus, both the survivin siRNA–induced arrest of cells in the more radiosensitive G2-M phase and the impaired post-irradiation DNA damage repair, may well have contributed to the induction of apoptosis through caspase-dependent or -independent pathways in otherwise resistant tumor cells against radiation. These findings are also in good correlation with the observed reduction in the slope of the survival curve shoulder and the increased ratio of $\alpha/\beta$ variables, indicative for a reduced sublethal or potentially lethal DNA damage repair in survivin siRNA–treated SW480 and HCT-15 cells after irradiation (Fig. 5; Table 1).

In summary, our results suggest that survivin, as determined in colorectal cancer patients likely to respond to conservative radiotherapies and that cancer Research.

Figure 6. Cumulative incidence plot of local relapse in 59 patients with advanced rectal cancer treated uniformly with a preoperative combination of radio- and chemotherapy and surgical resection, according to a high-survivin expression or low-survivin expression as determined in the pretreatment biopsies.

Table 1. Radiation response variables of SW480 and HCT-15 colorectal cells nontreated or transfected with survivin-specific siRNA

<table>
<thead>
<tr>
<th></th>
<th>Plating efficiency (%)</th>
<th>$\alpha$ (Gy$^{-1}$)</th>
<th>$\beta$ (Gy$^{-2}$)</th>
<th>LD$_{50}$ (Gy)</th>
<th>Radiation-induced cytotoxicity enhancement factor</th>
<th>LD$_{10}$ (Gy)</th>
<th>Radiation-induced cytotoxicity enhancement factor</th>
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<tr>
<td>SW480 Nontreated</td>
<td>39.6</td>
<td>0.36 ± 0.04</td>
<td>0.04 ± 0.09</td>
<td>1.62 ± 0.52</td>
<td>2.21 ± 0.74</td>
<td>4.32 ± 0.54</td>
<td>1.81 ± 1.08</td>
</tr>
<tr>
<td>+siRNA Surv</td>
<td>10.9</td>
<td>0.93 ± 0.08</td>
<td>0.01 ± 0.02</td>
<td>0.73 ± 0.07</td>
<td>2.39 ± 0.24</td>
<td>2.93 ± 0.25</td>
<td>1.52 ± 0.14</td>
</tr>
<tr>
<td>HCT-15 Nontreated</td>
<td>33.9</td>
<td>0.36 ± 0.01</td>
<td>0.03 ± 0.003</td>
<td>1.65 ± 0.05</td>
<td>1.75 ± 0.15</td>
<td>4.45 ± 0.16</td>
<td>1.52 ± 0.14</td>
</tr>
<tr>
<td>+siRNA Surv</td>
<td>20.9</td>
<td>0.70 ± 0.05</td>
<td>0.02 ± 0.01</td>
<td>0.94 ± 0.07</td>
<td>2.93 ± 0.25</td>
<td>2.93 ± 0.25</td>
<td>1.52 ± 0.14</td>
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NOTE: Radiation-induced cytotoxicity enhancement factors at 50% survival (LD$_{50}$) and 10% survival (LD$_{10}$) were calculated by transforming the linear quadratic equation ($SF = exp (-\alpha \times D - \beta \times D^2)$) using $\alpha$ and $\beta$ values of the individual survival curves.
gene therapeutic or pharmacological approaches targeting survivin in tumors overexpressing this protein could strongly increase the therapeutic ratio of radiotherapy for rectal cancer. The underlying mechanisms by which antisurvivin strategies may improve radiation response seem to be multifaceted, and involve suppression of caspase-mediated apoptosis as well as alterations in cell cycle distribution and impaired DNA double-strand break repair. Additional studies are ongoing to validate the optimal sequence of siRNA treatment during a fractionated course of radiotherapy in animal models of colorectal cancer.

References

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