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 intermediately 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).

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, Searing, 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′-CUG-GAC-AGA-GAA-GAG-GCC-ATT-3′; antisense, 5′-UGG-CUC-UUU-CUC-UUG-CCA-GTT-3′). Cells were treated in parallel with a green fluorescence protein (GFP)-siRNA (sense, 5′-GGU-CUG-UUG-GGA-GGU-CTT-3′; antisense, 5′-GAA-CUC-CAA-ACA-GCA-CAC-CTT-3′) as a nonspecific control (MWG, Ebersberg, Germany). Cells (5 × 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, irradiated cells were irradiated at room temperature using X-ray orthovoltage-irradiation (Stabilipan, Siemens, München, Germany) at 250 kV/m/15 mA/40 cm focus-surface distance at a dose rate of 1.15 Gy/min with single doses of 0, 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 sequences (77 bp), specific for all three known survivin transcripts, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts (96 bp) were amplified from cDNA in duplicate experiments by conventional PCR amplification and detection with an AmpliTaq rhodamin, deoxynucleotide triphosphates, and 1.25 units of AmpliTaq. Rather than mixing PCR 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 quencher 6-carboxytetramethylrhodamine. The fluorescence of the double-stranded siRNA (Eurogenetec, Searing, 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′-CUG-GAC-AGA-GAA-GAG-GCC-ATT-3′; antisense, 5′-UGG-CUC-UUU-CUC-UUG-CCA-GTT-3′). Cells were treated in parallel with a green fluorescence protein (GFP)-siRNA (sense, 5′-GGU-CUG-UUG-GGA-GGU-CTT-3′; antisense, 5′-GAA-CUC-CAA-ACA-GCA-CAC-CTT-3′) as a nonspecific control (MWG, Ebersberg, Germany). Cells (5 × 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, irradiated cells were irradiated at room temperature using X-ray orthovoltage-irradiation (Stabilipan, Siemens, München, Germany) at 250 kV/m/15 mA/40 cm focus-surface distance at a dose rate of 1.15 Gy/min with single doses of 0, 2, 4, 6, and 8 Gy.

**Cycle cell analysis.** Both adherent and detached SW480 and HCT-15 cells (1 × 10⁶/mL) were collected by trypsinization and washed with PBS for 10 minutes by centrifugation at 120 × g. Cells were resuspended in a staining solution containing 1 mg/mL propidium iodide, 4 mg/mL sodium citrate, 1 mg/mL RNase A (Boehringer Mannheim, Germany), and 0.1% Triton X-100. Fluorescence-activated cell sorting analysis was done with a Coulter EPICS flow cyrometer 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 Ser¹⁵-phosphorylated histone H2AX, cells were irradiated with 2 Gy, detached by accutase (PAAS, Pasching, Austria) treatment for 15 minutes, pelleted by centrifugation (5 minutes, 120 × g) and fixed in 70% ethanol 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 1:250 dilution or an isotype control antibody (both Upstate, Lake Placid, NY) for 2 hours in the dark. Cells were fixed in 1% parformaldehyde in PBS and analyzed using a Coulter EPICS-flow cytometer XL (Coulter). The mean fluorescence of the isotype-control was subtracted to eliminate unspecified 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 × (PE / 100), taking into consideration the individual plating efficiency (PE). Survival variables α and β were fitted according to the linear quadratic equation (SF = 1 − α × D − β × 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 with a 1:2000 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).
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.

Apoptotic 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 in SW480 and HCT-15 is mediated by short interfering RNA.** siRNA specific for survivin was used to transiently knock down its expression in SW480 and HCT-15 colorectal cell lines previously described to harbor high (SW480) and intermediately high (HCT-15) amounts of this antiapoptotic protein (7). To examine whether siRNA treatment alters mRNA levels of survivin in both lines, a quantitative TaqMan PCR was done from total RNA. Results are displayed in Fig. 1A. Twenty-four hours after transfection, the relative levels of survivin mRNA were decreased by 40% (SW480) and 20% (HCT-15), which were further reduced significantly (P < 0.001) at 48 hours (81% SW480, 73% HCT-15), and increased again at 72 hours (67% SW480, 40% HCT-15). Western blot analysis of total cellular extracts 24, 48, and 72 hours after transfection revealed a markedly reduced survivin protein expression in both SW480 and HCT-15 as compared with nontransfected or GFP-siRNA transfected control cells (Fig. 1A). 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

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**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, ± 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, ± 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, ± 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 $G_2$-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. 1\textsuperscript{A}) and protein (Fig. 1\textsuperscript{B}) expression by siRNA treatment resulted in an increased rate of spontaneous and radiation-induced apoptosis (Fig. 2\textsuperscript{A}) as well as an increased caspase 3/7 activity (Fig. 2\textsuperscript{B}). Transfection with survivin siRNA decreased cell viability (Fig. 3\textsuperscript{A}), and induced a G2-M arrest (Fig. 3\textsuperscript{B}). 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

![Figure 4. Increased phospho-histone γH2AX following treatment of survivin in SW480 colorectal cells. Displayed is the percentage of Ser\textsuperscript{139} phosphorylated-histone H2AX fluorescence following transfection with survivin-specific siRNA and irradiation with 2 Gy as assessed by FACS-analysis. Points, mean; bars, ± SD; *, \(P = 0.03\) versus nontreated controls.](image)

![Figure 5. Effect of survivin on the clonogenic survival of SW480 and HCT-15 cells transfected with either survivin-specific or GFP-control siRNA. Nontreated cells served as a control. Twenty-four hours later, the cells were irradiated with the indicated doses. After 12 to 14 days, colonies greater than 50 cells were counted and survival curves with survival fractions normalized to the plating efficiency were fitted according to the linear quadratic equation to determine survival parameters \(\gamma(Gy^{-1})\) and \(\delta(Gy^{-2})\). Points, mean; bars, ± SD.](image)
However, there also seemed to be additional mechanisms. First, we related cell death, probably via direct inhibition of caspases (21). Survival of tumor cells primarily through suppression of apoptosis-elicited. The conventional notion is that survivin enhances the clinical setting (17). The possible underlying mechanisms by which survivin may predictiv factor for radiation response and local control in the 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 rectal cancer treated uniformly with a preoperative combination of radio- and chemotherapy.

In summary, our results suggest that survivin, as determined in colorectal cancer are in accordance with reports in other malignancies showing an increased sensitivity of melanoma cells and lung cancer cells to irradiation following attenuation of survivin expression by ribozyme treatment and antisense, respectively (25–27). In this context, it is noteworthy that all the cell lines used within these studies harbor a mutated p53 gene. SW480 is described to harbor a G→A mutation in codon 273 of the p53 gene and a C>T mutation in codon 309 (28); HCT-15 is characterized by a heterozygous wild-type and C>G mutation at codon 153 (29). Therefore, although an interaction of the p53 protein and survivin a heterozygous wild-type and C > G mutation at codon 153 (29). and a C > T mutation in codon 309 (28); HCT-15 is characterized by a heterozygous wild-type and C > G mutation at codon 153 (29). Therefore, although an interaction of the p53 protein and survivin by direct promoter binding or more indirectly by chromatin deacetylation or enhanced p53 degradation has recently been described (30–32), the radiosensitizing effects of survivin inhibition did not seem to be mediated by wild-type p53. This is of relevance for the clinical setting, as mutated p53, found with a high incidence in rectal cancer, may not interfere with a putative therapeutic intervention of the survivin pathway in combination with radio- and chemotherapy.

In summary, our results suggest that survivin, as determined in pretreatment biopsies, could serve as a predictive factor to identify patients likely to respond to conservative radiotherapies and that
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 include 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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