XIAP as a Radioresistance Factor and Prognostic Marker for Radiotherapy in Human Rectal Adenocarcinoma

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A differential responsiveness of patients to ionizing radiation is observed after preoperative radiotherapy for rectal adenocarcinoma that might be related, in part, to an apoptosis defect. To establish if proteins of the apoptotic cascades [pro-apoptotic: active caspase 3, 8, and 9 and DIABLO (direct inhibitor of apoptosis-binding protein with low pI); anti-apoptotic: XIAP (X-linked inhibitor of apoptosis)] are involved, we analyzed their profile in radioresistant (SW480) and radiosensitive (SW48) human colorectal cell lines. We demonstrated that, after irradiation, the SW48 cells increased the expression of the pro-apoptotic proteins, whereas the SW480 cells increased the expression of the anti-apoptotic protein XIAP. Moreover, XIAP knockdown in SW480 cells enhanced the basal and radiation-induced apoptotic index; the propensity of the SW480 cells to undergo apoptosis after radiation was higher compared with SW48 cells. In a translational study of 38 patients with rectal carcinoma, we analyzed the apoptotic profile for tumor and noncancerous tissue for each biopsy specimen using IHC. According to their response to preoperative radiotherapy, patients were classified into two groups: responsive and nonresponsive. Although no difference in expression of caspase 3, 8, or 9 was observed in the tumor/normal tissue ratio between responsive and nonresponsive patients, the ratio decreased for DIABLO and increased for XIAP. In conclusion, inhibition of XIAP rescues cellular radiosensitivity and both DIABLO and XIAP might be potential predictive markers of radiation responsiveness in rectal adenocarcinoma. (Am J Pathol 2012, 181:1271–1278; http://dx.doi.org/10.1016/j.ajpath.2012.06.029)
expression in individual lesions. Although it is possible to identify patients with a radioresponsive tumor at the time of diagnosis, a selective and individualized policy of preoperative radiotherapy as well as less radical surgery might be pursued.

Among the molecular mechanisms potentially involved in the differential response of rectal carcinoma to radiation, an association has been observed between the apoptotic rate and the radiosensitivity of the tumor. Moreover, either by genome-wide candidate gene approaches, several apoptotic proteins [BCL2, BAX, pro-caspase (CASP), and Survivin] or apoptosis-related genes (TP53 and p21) have been described as being involved in the response of rectal carcinoma to radiation, suggesting that apoptotic cell death may play an important role in determining the response to radiotherapy. However, the role of caspases (through their active form) and the involvement of a different type of proteins of the executioner step of apoptosis remain to be elucidated. This step is under the control of cytochrome proteases, named effectors CASP3, CASP6, and CASP7, that are activated by initiator caspases, such as active CASP8 and CASP10, for the death receptor pathway, and active CASP9 for the mitochondrial pathway. Effector caspase activity, in turn, is controlled by members of the inhibitor of apoptosis protein family (IAP). Baculoviral IAP repeat-containing (BIRC) 4X-linked inhibitor of apoptosis (XIAP) is the most thoroughly characterized IAP. IAPs bind to and inhibit caspase activation and/or activity. Furthermore, apoptosis-promoting proteins located inside the mitochondria, such as direct inhibitor of apoptosis-binding protein with low pi (DIABLO), are antagonized by the caspase-inhibiting effects of XIAP. Overall, the relative levels of the three types of proteins (effector caspases, IAPs, and inhibitors of IAPs) will preside over the apoptotic fate of the cells.

In our study, we aimed to establish the involvement of active CASPs 3, 8, and 9, DIABLO, and XIAP in the radioresistance process by comparing their profile in radioresistant (SW480) and radiosensitive (SW48) human colon cell lines and to assess the effects of knockdown of XIAP on cell line response to ionizing radiation. To confirm the in vitro data at the clinical level, we analyzed the expression of these proteins in rectal carcinoma tissues from patients characterized as responsive or nonresponsive to preoperative radiotherapy by a semiquantitative immunohistochemical (IHC) approach.

Materials and Methods

Cell Culture and Irradiation Procedure

Human colorectal adenocarcinoma cell lines SW48 and SW480, with diverse and characterized intrinsic radiosensitivities, were obtained from ATCC (LGC-Promochem, Wiesbaden, Germany). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% sodium pyruvate, and 2 mmol/L glutamine and seeded at a density of 15,000 cells/cm². The cells were transfected (20 to 50 nmol/L) with a small-interfering RNA (siRNA; MWG Biotech AG, Washington, DC) raised against Xip (5′-UGCAA-GAGCGUGGAUUAUGCTT-3′) or a nonspecific scramble siRNA (5′-UGGGACUAAGUUAUGAUCGUCTT-3′) or incubated with the Interferin Transfection reagent (Polyplus-Transfection SA, Illkirch, France) alone for 48 hours. Next, fresh culture medium was added to cells before being irradiated with a single dose of 2 or 5 Gy, with a dose rate of 20.44 mGy/s (Clinac 2100/CD; VARIAN Medical Systems, Palo Alto, CA).

 Colony-Forming Assay

The clonogenic colony-forming assay was performed as previously described. Briefly, SW48 or SW480 cells were transfected with either XIAP-specific siRNA or nonspecific scrambled siRNA or incubated with the transfection reagent, as described in “Cell Culture and Irradiation Procedure”. For colony-forming assays, cells were plated as single cells into six-well plates (BD, Heidelberg, Germany) in triplicate, 24 hours after transfection. After an additional 24-hour incubation at 37°C and 5% CO₂, cells were irradiated at room temperature with single doses of X-ray (0, 2, or 6 Gy). Colonies were stained with methylene blue solution for 30 minutes, and colonies (>50 cells) were counted subsequently at 19 (SW48) or 13 (SW480) days after plating.

Calculation of surviving fractions was performed according to the following equation:

Surviving Fraction = Colonies Counted/Cells Seeded × (Plating Efficiency/100), considering the individual plating efficiency.

Survival variables α and β were fitted according to the following linear quadratic equation: Surviving Fraction = exp ((−α × D) − (β × D²)), with D = dose, using EXCEL software (Microsoft, Unterschleißheim, Germany).

All experiments were repeated three times. Radiation-induced cytotoxicity enhancement factors at 50% and 10% survival were calculated by transforming the previously mentioned equation using α and β values of the individual survival curves.

Apooptotic Assays

The apoptotic rate in SW48 and SW480 cell lines was assessed using flow cytometric detection of annexin V–fluorescein isothiocyanate (Mitenyi Biotec GmbH, Gladbach, Germany), according to the instructions of the manufacturer. CASP3 activity was measured in SW48 and SW480 cell lines using a colorimetric assay kit (Alexis Biochemicals, Paris, France), according to the manufacturer’s instructions.

Western Blot Analysis

SW48 and SW480 cells were homogenized in 200 μL of ice-cold hypotonic buffer (25 mmol/L Tris-HCl, pH 7.4, containing protease inhibitor cocktail). Protein concentration was determined using the bicinchoninic acid assay. Proteins (5 to 10 μg) were separated on 10% polyacrylamide gels and transferred to nitrocellulose
membranes.25 The membranes were incubated overnight at 4°C with the primary antibodies (see IHC Data), and equal protein loading was confirmed by reprobing the blot with a rabbit IgG anti-actin antibody (1:600; Sigma-Aldrich, L’Isle D’Abeau, France). The antigen-antibody complexes were visualized by using a chemiluminescent kit (Covalight; Covalab, Lyon, France). Biomax MR films (Sigma) were scanned, and the estimated intensity of the bands was quantified using the Optiquant software package (Packard, Meriden, CT).

Preoperative Radiochemotherapy Protocol

A cohort of 38 patients with locally advanced rectal carcinoma received preoperative radiochemotherapy before surgery using a total dose of 45 Gy in daily fractions of 1.8 Gy on 5 consecutive days each week. During the first and fifth weeks of radiotherapy, 5-fluorouracil was delivered at a dose of 350 mg/m² per day; and folic acid was delivered at a dose of 20 mg/m². The TNM was assessed by ultrasonography before radiotherapy, and the ypTNM staging was determined based on the surgical specimens. (ypTNM describes the anatomic extent of disease after surgery.) Two groups of patients (patients who were responsive and nonresponsive to radiotherapy) were determined by comparing the ultrasonographic TNM with the ypTNM. After treatment, the tumor stage grouping (ypTNM) in the responsive group was changed in all patients [19 (100%) of 19] because no more tumoral tissue was observed (complete regression). In the nonresponsive group, 19 patients, the ypT stage was decreased for 7 (36.8%), unchanged for 10 (52.6%), and increased for the remaining 2 (10.5%). Moreover, in the 19 patients, the ypN stage was decreased in 9 (47.4%), unchanged in 6 (31.6%), and increased in 4 (21.1%). The ethics committee of the medical faculty and the state medical board agreed to these investigations and an informed consent was obtained from all patients.

IHC Data

Tissues were immediately fixed for 24 hours in Bouin’s fluid and handled as previously described.21 The Envision+ kit (Dako, Trappes, France) was used with anti-TP53 (1:800; BD Biosciences, Le Pont de Clai, France), anti-DIABLO (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-cleaved CASP8 (1:150; Ozyme, Saint Quentin Yvelines, France) antibodies. The CSAll (Biotin-Free Tyramide Signal Amplification) system (Dako) was used with anti-cleaved CASP9 antibodies (1:400; Clini-sciences, Montrouge, France), and an LSAB kit (Dako) was used with anti-XIAP (1:80; BD Biosciences) and anti-cleaved CASP3 (1:80; Ozyme) antibodies. Two independent pathologists (M.D. and D.M.), blinded to the clinicopathologic variables, evaluated the immunostaining. The immunostaining score was evaluated based on the whole slide. The results were recorded by assessing the percentage of positive cells and the intensity of the staining (1, mild; 2, moderate; 3, intense) on the tumoral and the nontumoral areas for each slide, as previously described.26 Immuno-staining scores were calculated by multiplying the percentage of labeled cells by the intensity of staining.

Data Analysis

In vitro experiments were realized in quadruplicate and were performed at least three times in independent experiments. A one-way analysis of variance for dependent groups was performed to determine whether there were differences between all groups, followed by a Bonferroni post hoc test, if \( P < 0.05 \) in the analysis of variance, to determine the significance (\( P < 0.05 \)) of differences between pairs of groups. A Mann-Whitney test was used to compare the expression in tumor and nontumoral tissue and to compare the ratio for tumoral/nontumoral tissue between responsive and nonresponsive groups of patients. \( P < 0.05 \) was considered significant. The statistical tests were performed using StatView software, version 5.0 (SAS Institute Inc., Cary, NC).

Results

Effects of Ionizing Radiation on SW480 and SW48 Cell Survival and Apoptotic Profile

The radiation response characteristics of both cell lines were investigated using apoptotic cell death evaluation (see Supplemental Figure S1 at http://ajp.amjpathol.org). Ionizing radiation induced a significant increase in the number of apoptotic SW48 cells at 2 Gy (4.6-fold, \( P < 0.002 \)) and 5 Gy (6.8-fold, \( P < 0.001 \)). Although irradiation induced a significant increase of apoptotic SW480 cells at 2 Gy (1.7-fold, \( P < 0.006 \)) and 5 Gy (2.0-fold, \( P < 0.001 \)), such an increase was two to three times less potent than in SW48 cells. Similar results were obtained with the kinetic study, confirming that the SW48 cell line was more sensitive to ionizing radiation than SW480 cells. To further analyze the diverse intrinsic radiation responses of these cell lines, we evaluated their apoptotic profile. In the SW48 cell line, irradiation induced a significant increase in active CASP8 protein levels at 2 Gy (1.5-fold, \( P < 0.0001 \), Figure 1A) and 5 Gy (4.3-fold, \( P < 0.0009 \), Figure 1B). By contrast, irradiation of the SW480 cell line induced a significant decrease in active CASP8 protein levels at 2 Gy (5.3-fold, \( P < 0.0009 \), Figure 1A) and 5 Gy (5.3-fold, \( P < 0.0001 \), Figure 1B). Irradiation of SW48 cells induced a significant increase in CASP3 activity at 2 Gy (1.7-fold, \( P < 0.01 \), Figure 1C) or 5 Gy (2.1-fold, \( P < 0.0009 \), Figure 1D), whereas irradiation of the SW480 cell line did not modify the CASP3 activity at 2 Gy (Figure 1C) but induced a significant increase at 5 Gy (1.4-fold, \( P < 0.03 \), Figure 1D). For XIAP, irradiation of the SW48 cell line did not alter its protein expression with either 2 Gy (Figure 2A) or 5 Gy (Figure 2B). Irradiation of the SW480 cell line induced a significant increase in XIAP protein levels at 2 Gy (1.5-fold, \( P < 0.0003 \), Figure 2A) and 5 Gy (1.5-fold, \( P < 0.0003 \), Figure 2B). The expression of DIABLO was unchanged in SW48 cells irradiated at 2 Gy (Figure 2C) and 5 Gy (Figure 2D). Irradiation of the SW480 cell line induced a significant decrease in
DIABLO protein levels at 2 Gy (4.9-fold, \( P < 0.0001 \), Figure 2C) or 5 Gy (approximately 50-fold, \( P < 0.0004 \), Figure 2D). These data suggested that the anti-apoptotic molecule XIAP and DIABLO (the XIAP inhibitor) are indicators for the differential radiation response of SW48 and SW480 cells.

**XIAP Knockdown Radiosensitized SW48 and SW480 Cells**

Under our experimental conditions, siRNA knockdown of XIAP decreased its protein levels by 98%, whereas the transfectant reagent had no effect (Figure 3, A and C). Under nonirradiated or irradiated conditions, the apoptotic fraction for the transfectant reagent was similar to mock-treated cells (Figure 2E). XIAP knockdown induced a significant increase in the apoptotic rate of SW48 (1.6-fold, \( P = 0.018 \)) and SW480 (threefold, \( P = 0.001 \)) cells compared with mock-treated cells (Figure 2E). Moreover, knockdown of XIAP, if combined with ionizing radiation, induced a fourfold \( (P < 0.002, \text{SW48}) \) or a 5.3-fold \( (P < 0.002, \text{SW480}) \) increase in the apoptotic rate when compared with mock-treated cells (Figure 2E). Finally, the association of XIAP knockdown plus irradiation enhanced the apoptotic rate of SW480 cells, which reached a significantly higher level than the apoptotic rate of SW48 cells (1.3-fold, \( P < 0.008 \), Figure 2E).

To further establish a correlation between XIAP attenuation and radiation response, clonogenic survival assays were performed. Pre-incubation of SW48 cells (Figure 3A) and SW480 cells (Figure 3C) with XIAP siRNA slightly, but not significantly, increased basic clonogenic survival compared with the mock-treated controls. By contrast, XIAP siRNA transfection significantly radiosensitized SW48 (Figure 3B) and SW480 (Figure 3D) cells, resulting in a calculated radiation-induced cytotoxicity enhancement factor of 1.34 (LD50) or 1.19 (LD10) for SW48 and 1.33 (LD50) or 1.11 (LD10) for SW480 (Table 1).

**In Vivo Apoptotic Profile in Human Rectal Carcinoma Tissues**

We performed a semiquantitative IHC analysis for the different apoptotic molecules studied to evaluate if an alteration in the apoptotic profile might explain the radiation responsiveness of patients with rectal adenocarcinoma (see Supplemental Figure S2 at http://ajp.amjpathol.org). For each protein studied, we compared the following: its expression between the tumoral and circumjacent noncancerous tissue and the ratio for responsive/nonresponsive patients. The expression of active CASP8 (Figure 4A), active CASP9 (Figure 4B), and active CASP3 (Figure 4C) was significantly higher in tumors compared with noncancerous tissue in both responsive \( (P < 0.003, \)
CASP8; P < 0.002, CASP9; P < 0.001, CASP3) and nonresponsive (P < 0.009, CASP8; P < 0.002, CASP9; P < 0.01, CASP3) groups. By contrast, the ratio for tumor/noncancerous tissue was unchanged for active CASP8 (Figure 4A), CASP9 (Figure 4B), and CASP3 (Figure 4C). The expression of XIAP (Figure 4D) was significantly higher in the tumor tissue compared with the noncancerous tissue in both responsive (P < 0.003) and nonresponsive (P < 0.03) groups. Notably, the ratio for tumor/noncancerous tissue for XIAP was higher in the nonresponsive compared with the responsive group (P < 0.04, Figure 4D). The expression of DIABLO was significantly elevated in tumors only of the responsive group (P < 0.002, Figure 4E). The ratio for tumoral/nontumoral tissue was lower in the nonresponsive group for DIABLO (P < 0.02, Figure 4E). Because the TP53 status correlated to the radiation response of colorectal cancer,27 we evaluated its expression. Although TP53 protein levels were significantly higher in tumoral compared with nontumoral tissue in both responsive (P < 0.001) and nonresponsive (P < 0.001) groups, the ratio of tumoral/nontumoral tissue was similar in both groups (Figure 4F).

### Table 1. Radiation Response Variables of XIAP Knockdown and Control SW48 and SW480 Cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Plating efficiency (%)</th>
<th>α (Gy⁻¹)</th>
<th>β (Gy⁻²)</th>
<th>LD₁₀ (Gy)</th>
<th>Radiation enhancement ratio LD₁₀</th>
<th>Radiation enhancement ratio</th>
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</thead>
<tbody>
<tr>
<td>SW48</td>
<td>C</td>
<td>29.81 ± 2.38</td>
<td>0.1722</td>
<td>0.0925</td>
<td>1.96</td>
<td>4.14</td>
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<tr>
<td></td>
<td>Si0</td>
<td>29.48 ± 1.61</td>
<td>0.1959</td>
<td>0.0809</td>
<td>1.96</td>
<td>4.26</td>
<td></td>
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<tr>
<td></td>
<td>SiX</td>
<td>34.53 ± 6.35</td>
<td>0.3601</td>
<td>0.0799</td>
<td>1.46</td>
<td>1.34</td>
<td>3.57</td>
</tr>
<tr>
<td>SW480</td>
<td>C</td>
<td>29.62 ± 2.06</td>
<td>0.0260</td>
<td>0.0498</td>
<td>3.48</td>
<td>6.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Si0</td>
<td>34.42 ± 6.46</td>
<td>0.0673</td>
<td>0.0405</td>
<td>3.39</td>
<td>6.75</td>
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</tr>
<tr>
<td></td>
<td>SiX</td>
<td>35.78 ± 7.55</td>
<td>0.1966</td>
<td>0.0298</td>
<td>2.54</td>
<td>1.33</td>
<td>6.09</td>
</tr>
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</table>

Radiation-induced cytotoxicity enhancement factors at 50% (LD₁₀) and 10% (LD₁₀) survival were calculated by transforming the following linear quadratic equation: Surviving Fraction = exp [(-α × D) – (β × D²)], with α and β values of the individual survival curves.  

- **C**, cells treated only with transfection reagent;  
- **Si0**, transfection with nonspecific scrambled siRNA;  
- **SiX**, transfection with XIAP-specific siRNA.
Discussion

Preoperative radiotherapy of rectal adenocarcinoma induces a heterogeneous tumor response. Moreover, the rate of complete pathological response induced by chemotherapy, classically given in combination with ionizing radiation, remains wide (9% to 37%), per a review. A better understanding of the molecular basis of differential radiation responses of rectal adenocarcinoma might open the possibility for identifying a predictive marker to detect responsive and nonresponsive patients. Moreover, the use of a specific molecule, targeted according to the radiosensitivity status, might enhance preoperative radiotherapy efficacy and reduce adverse effects. A high level of apoptotic tumor cells is a predictive marker for tumor response and long-term local control in colorectal cancer, and a correlation between radiation-induced apoptosis and radiosensitivity has been identified in vitro. However, the apoptotic cascade proteins involved in the differential response of patients to radiotherapy have not been elucidated.

In that context, we compared the apoptotic profile after irradiation of known radio-sensitive (SW48) and radioreistant (SW480) cell lines. It is well established that TP53 is involved in regulating radiation-induced apoptosis in colorectal cancer. However, the radiation response of the TP53 mutated cell lines is controversial. In the case of the colorectal cell lines used in our study, SW48 cells carry the R248W TP53 point mutation, whereas the SW480 cells carry both R273H and P309S point mutations. Whether the point mutations (specifically, the R273H mutation) carried by the SW480 cell line enhance its radiation response status remains an open question. Indeed, R273H mutated cell lines show either higher radioresistance or no difference in radiosensitivity. Moreover, Jurkat cells transfected with R273H-mutated TP53 show no difference in their sensitivity compared with control cells. However, the levels of mutated TP53 protein in SW480 cells are approximately 20-fold higher than in the strain LoVo that expresses wild-type protein.

We observed high levels of pro-apoptotic molecules in the radiosensitive SW48 cells and low levels of pro-apoptotic molecules in the radioreistant SW480 cells. Moreover, we observed increased protein levels of the anti-apoptotic factor XIAP in SW480 cells. XIAP has a functional role, because inhibition of its expression in the radioreistant (SW480) cells increased their apoptotic rate in both basal (nonirradiated) and irradiated conditions. To associate these preclinical findings at the clinical level, we used a semiquantitative IHC approach to analyze the expression of the caspases in 38 biopsy specimens obtained from 19 responsive and 19 nonresponsive patients with rectal cancer treated with preoperative chemoradiation. Given that the expression of active CASP3, CASP8, and CASP9 was increased in tumor tissue when compared with nontumorous tissue, for both responsive and nonresponsive groups, but without any observed modification in the ratio of tumoral/nontumoral tissue between the two groups, we hypothesized that these molecules might not represent markers for radiation response in rectal carcinoma. However, although high levels of active CASP8, CASP9, and CASP3 (pro-apoptotic proteins) were expressed in tumoral tissue compared with nontumorous tissue, the apoptotic index remained low in tumor cells, suggesting a blockade in the cell death process in rectal adenocarcinomas. One putative mechanism underlying this blockade is a deregulation in the expression of XIAP and its inhibitor, DIABLO. We observed an increase for XIAP in the ratio for tumoral/nontumoral tissue in nonresponsive compared with responsive patients. Conversely, the ratio was dramatically decreased for DIABLO in the nonresponsive patient group. Alternately, increased XIAP levels may down-regulate CASP3 and CASP9 activities, whereas decreased DIABLO levels may permit XIAP [and cellular (c)IAP1 and cIAP2] action as anti-apoptotic factors. Thus, the ratio for XIAP and DIABLO might represent a biomarker for radiation responses in rectal cancer. An overexpression of DIABLO in a cervical cancer cell line enhanced the expression and activity of CASP3 and improved the apoptosis-inducing effects of radiation on these cells. XIAP might have an important role in colorectal cancer development and progression. Indeed, XIAP mRNA was significantly higher in colorectal cancer tumors than in normal mucosa or colorectal adenoma, and XIAP levels were correlated with the pathological stage of colorectal cancer. More widely, XIAP might represent a marker for malignancy, because it is highly expressed in most human cancer cells. Notably, Survivin, another member of the IAP family, was also a biomarker in patients with rectal cancer. Increased Survivin levels were associated with an increased risk of local tumor recurrence and knockdown of Survivin expression in the radioreistant cell line SW480 or in tumor xenografts, resulting in an increased apoptotic fraction or delay in tumor growth after radiation. The novel finding of our study is the specific differential profile of XIAP and DIABLO expression in the nonresponsive patients. The ratio was obtained by comparing tumoral with nontumorous tissues from the same patients, therefore bypassing individual variation. Moreover, XIAP might be a promising target for molecular tumor therapy. The ability to selectively down-regulate XIAP by the siRNA approach has enabled us to highlight the importance of this IAP in resistance to radiotherapy-induced cell death in colorectal carcinoma cells. Indeed, several therapeutic tools in preclinical trials have been developed to inhibit XIAP expression or function, such as antisense oligonucleotides or small molecules that mimic DIABLO action. These molecules may be new anticancer drugs that mediate down-regulation of XIAP and/or restoration of DIABLO activities in vivo and synergize disease-relevant therapies. Moreover, because high levels of pro-apoptotic molecules (eg, active CASP8) are observed in human rectal carcinoma, sensitization effects of XIAP suppression and/or DIABLO restoration may be augmented by using tumor necrosis factor-related apoptosis-inducing ligand treatment therapeutical tools.

In summary, our results suggest the following: XIAP and DIABLO evaluated in tumoral versus nontumoral tissues from rectal biopsy specimens before treatment could serve as a biomarker to identify patients likely to
either respond or not respond to preoperative radiotherapy and XIAP might be targeted to increase the therapeutic ratio of radiotherapy for rectal cancer.

Acknowledgment

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