Prognostic Significance of TRAIL Signaling Molecules in Stage II and III Colorectal Cancer

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Abstract

Purpose: We previously found that cellular FLICE-inhibitory protein (c-FLIP), caspase 8, and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) receptor 2 (DR5) are major regulators of cell viability and chemotherapy-induced apoptosis in colorectal cancer. In this study, we determined the prognostic significance of c-FLIP, caspase 8, TRAIL and DR5 expression in tissues from patients with stage II and III colorectal cancer.

Experimental Design: Tissue microarrays were constructed from matched normal and tumor tissue derived from patients (n = 253) enrolled in a phase III trial of adjuvant 5-fluorouracil–based chemotherapy versus postoperative observation alone. TRAIL, DR5, caspase 8, and c-FLIP expression levels were determined by immunohistochemistry.

Results: Colorectal tumors displayed significantly higher expression levels of c-FLIP (P < 0.001), caspase 8 (P = 0.01), and DR5 (P < 0.001), but lower levels of TRAIL (P < 0.001) compared with matched normal tissue. In univariate analysis, higher TRAIL expression in the tumor was associated with worse overall survival (P = 0.026), with a trend to decreased relapse-free survival (RFS; P = 0.06), and higher tumor c-FLIP expression was associated with a significantly decreased RFS (P = 0.015). Using multivariate predictive modeling for RFS in all patients and including all biomarkers, age, treatment, and stage, we found that the model was significant when the mean tumor c-FLIP expression score and disease stage were included (P < 0.001). As regards overall survival, the overall model was predictive when both TRAIL expression and disease stage were included (P < 0.001).

Conclusions: High c-FLIP and TRAIL expression may be independent adverse prognostic markers in stage II and III colorectal cancer and might identify patients most at risk of relapse. Clin Cancer Res; 16(13); 3442–51. ©2010 AACR.

Colorectal cancer is the second most common cause of cancer-related deaths. It is now well established that in patients with stage III disease undergoing curative surgical resection, adjuvant 5-fluorouracil (5-FU)–based chemotherapy reduces tumor recurrence rates and improves overall survival (1–3). However, as many as 65% of patients with stage III colorectal cancer are cured by surgery alone (4). In stage II disease, the QUASAR trial, comparing adjuvant chemotherapy versus observation alone, concluded that although some patients benefited from adjuvant therapy, the improvement in 5-year survival was small (3.6%), and >80% of stage II patients were cured by surgery alone (2). Thus, the identification and validation of prognostic biomarkers of relapse in stage II and III colorectal cancer are urgently needed to spare patients, who could be cured by surgery alone, from unnecessary treatment with chemotherapy. This has become even more important following the addition of oxaliplatin to a fluoropyrimidine as an option in adjuvant therapy, as there is now a higher potential for longer term toxicity such as sensory neuropathy.

The aim of this study is to use immunohistochemistry to determine the expression patterns of cellular FLICE-inhibitory protein (c-FLIP), caspase 8, tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), and TRAIL receptor 2 (DR5) in matched normal and tumor epithelium in patients with stage II/III colorectal cancer enrolled in an adjuvant trial comparing postoperative...
Translational Relevance

Deregulated apoptotic signaling is a hallmark of cancer. In previous studies, we identified cellular FLICE-inhibitory protein (c-FLIP), caspase 8, and the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor, DR5, as important regulators of colorectal cancer cell survival, both constitutively and in the context of chemotherapeutic treatment. In this study, we have analyzed the expression of c-FLIP, caspase 8, TRAIL, and DR5 in matched normal and tumor tissues from a cohort of colorectal cancer patients enrolled in a phase III trial of adjuvant 5-fluorouracil–based chemotherapy versus observation alone. Colorectal tumors expressed significantly higher levels of c-FLIP, caspase 8, and DR5 but lower levels of TRAIL compared with matched normal tissue, suggesting that altered expression of these proteins might be important in the pathogenesis of this disease. Importantly, we found that high c-FLIP and TRAIL expression were independent adverse prognostic markers that could identify patients most at risk of relapse and who therefore would most benefit from adjuvant chemotherapy treatment.

5-FU–based chemotherapy versus observation alone and to correlate expression with clinicopathologic variables and outcome. Deregulated apoptosis is a hallmark of cancer (5). Apoptosis can be divided into two main pathways: the death receptor (DR)–mediated (or extrinsic) pathway and the intrinsic mitochondrial-regulated pathway (6). The extrinsic apoptotic pathway is regulated via specialized cell surface receptors, which are members of the tumor necrosis factor (TNF) receptor family, including TNFR1, Fas, and the TRAIL receptors, DR4 and DR5. Although Fas ligand and TNF-α are too toxic to be used as systemic therapies, TRAIL and agonistic antibodies targeting DR4 and DR5 have received much attention as potential therapeutic death ligands due to their high level of tumor specificity (7–10). Therefore, the relative expression of TRAIL signaling molecules in normal and tumor colorectal tissues is now a highly relevant issue.

A key inhibitor of DR-mediated apoptosis is c-FLIP, which inhibits caspase 8 processing at the death-inducing signaling complexes formed by these receptors (11). Differential splicing gives rise to long (c-FLIPL) and short (c-FLIPS) forms of c-FLIP (12). Death-inducing signaling complex–bound c-FLIP has also been reported to promote the activation of multiple pro-survival signaling pathways (13, 14). We have previously found that c-FLIP is a major regulator of viability and chemotherapy-induced apoptosis in colorectal cancer cells in vitro and in vivo due to its ability to block apoptosis mediated by caspase 8 and DR5 (15–17). These results, and the advent of TRAIL-targeted therapeutics, make it highly important to determine the relative expression of c-FLIP, caspase 8, and DR5 in normal and tumor colorectal tissues. In addition, two previously published studies identified c-FLIP expression as an independent adverse prognostic marker for disease outcome in colorectal cancer (18, 19). We sought to verify and extend these findings in the current study as we had access to paired normal and tumor specimens and equal numbers of untreated and chemotherapy-treated stage II and III patients. Moreover, there have been conflicting reports on the prognostic significance of the expression of TRAIL and DR5 in colorectal cancer, which we sought to resolve (20, 21). In addition, although caspase 8 has been reported to be overexpressed in colorectal (22) and rectal cancer (23), to our knowledge, caspase 8 protein expression has not previously been correlated with disease outcome.

Materials and Methods

Patient details

This study was based on a phase III randomized trial involving 253 patients accrued between 1994 and 1997 with stage II and III colorectal cancer. Following surgical resection, these patients were randomized to either observation alone or adjuvant treatment with bolus 5-FU/folinic acid (24). Matched normal and tumor tissues were obtained from the same patient. Prior to use, resected tissues were conserved as formalin-fixed tissue embedded in paraffin in accordance with routine diagnostic histopathology practice. At the time of study analysis, the median follow-up was 6.5 years. The clinical and pathologic details of these patients are displayed in Table 1. The CONSORT diagram (Supplementary Fig. S1) displays the number of patients involved in each arm. There was full approval from the local research ethics committee and all involved hospitals, and all patients gave consent for the use of their specimens in research, according to the Declaration of Helsinki.

Tissue microarray construction

Four 0.6-mm cores were obtained from both the normal and tumor tissue of each patient using a Beecher Instruments arrayer and placed into a paraffin block. Sections 4 μm in thickness were cut, floated onto adhesive slides, and baked overnight at 55°C. Arrays were constructed at a density of 90 to 110 cores per array.

Immunohistochemical detection methods

Details for the primary antibodies used are displayed in Supplementary Table S1. Initially, antibodies that were reported to stain formalin-fixed, paraffin-embedded tissues were selected and tested using relevant positive control tissues (either recommended by the antibody manufacturer or selected from previous publications). For each antibody, a series of optimization steps were done to determine the most appropriate antigen retrieval protocol and antibody dilution to use. This optimization was based on the staining pattern obtained in the positive control ensuring that...
the correct areas were stained intensely with background staining kept to a minimum. All staining was done on a BondMax automated immunostainer (Vision BioSystems). Briefly, for all markers except c-FLIP, following appropriate pretreatment, the diluted primary antibody was applied to the cores for 20 minutes, washed in buffer, and then endogenous peroxidase was blocked via 3% hydrogen peroxide.

Table 1. Clinicopathologic details of colorectal cancer patient cohort (N = 253)

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>5-FU/folinic acid, n = 126 (%)</th>
<th>Observation, n = 127 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>Range</td>
<td>38-81</td>
<td>35-80</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>71 (56)</td>
<td>79 (62)</td>
</tr>
<tr>
<td>Female</td>
<td>55 (44)</td>
<td>48 (38)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>79 (63)</td>
<td>81 (64)</td>
</tr>
<tr>
<td>III</td>
<td>47 (37)</td>
<td>46 (36)</td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right colon</td>
<td>37 (29)</td>
<td>32 (25)</td>
</tr>
<tr>
<td>Left colon</td>
<td>50 (40)</td>
<td>59 (47)</td>
</tr>
<tr>
<td>Rectum</td>
<td>35 (28)</td>
<td>36 (28)</td>
</tr>
<tr>
<td>Synchronous</td>
<td>4 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Grade of differentiation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>107 (85)</td>
<td>107 (84)</td>
</tr>
<tr>
<td>High grade</td>
<td>15 (12)</td>
<td>15 (12)</td>
</tr>
<tr>
<td>Not specified</td>
<td>4 (3)</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Vascular invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>67 (53)</td>
<td>69 (54)</td>
</tr>
<tr>
<td>Yes</td>
<td>27 (22)</td>
<td>25 (20)</td>
</tr>
<tr>
<td>Not specified</td>
<td>32 (25)</td>
<td>33 (26)</td>
</tr>
</tbody>
</table>

For c-FLIP, the sections were dewaxed and antigen retrieval done on the BondMax as above, with subsequent primary antibody incubation occurring overnight at 4°C. Antibody detection was then carried out as above using the Bond Polymer Refine kit, an enhanced diaminobenzidine as chromagen, and Harris hematoxylin as counterstain.

Scoring

Cores were evaluated by two independent observers (D. McLornan and H. Barrett) blinded to clinical data. The scoring field was ×100 (×10 eyepiece with ×10 objective), and the whole of each tissue was scored. Staining intensity was graded as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). Staining extent was graded from 0 (0-5% positive epithelial cells), 1 (6-25% positive), 2 (26-50% positive), 3 (51-75% positive), and 4 (>75% positive). The intensity and the extent of the scores were multiplied for each core, and both the mean normal and mean tumor expression signature score for a particular immunohistochemical marker calculated. Any discordance between scores was agreed by consensus.

Statistics

Patient data was collected via a centralized trial-coordinating office and stored electronically. Statistical analysis was done using the SPSS 13 software package (SPSS, Inc.). Comparisons between matched normal and malignant tissues were done via matched Student's t tests. Correlations were assessed via Pearson's correlation coefficient method. Survival analysis was done using Kaplan-Meier and Cox regression proportional analyses for overall survival and relapse-free survival (RFS). Multivariate predictive modeling was done using the stepwise backward log rank method of Cox regression analysis. Follow-up was from the date of surgery.

Results

Patient cohort characteristics

A tissue microarray was constructed using tissue samples from patients entered into a phase III randomized controlled trial of treatment with 5-FU/folinic acid chemotherapy versus observation alone in patients with surgically resected stage II and III tumors (24). Matched normal and tumor tissue samples were arrayed for each of the 254 patients recruited to the trial from hospitals throughout Northern Ireland between 1994 and 1997. The patients’ characteristics are shown in Table 1. Two-thirds of the patients had stage II disease. Age, gender, stage, and grade of tumor were well matched between the two study arms. One patient was ineligible as a result of undiagnosed metastatic disease found to have been present at study entry.

Survival analysis: effect of stage and treatment

Analysis of survival was carried out when every patient had undergone at least 5 years of follow-up. Figure 1A depicts the overall survival for the two study arms. There was no statistically significant improvement in survival in the chemotherapy arm of the study (P = 0.26; Fig. 1A). As expected, there was a highly significant difference in overall survival when patients were analyzed according to their tumor stage (P < 0.001; Fig. 1B). The trend towards improved survival with chemotherapy was present for both stage II and III patients, although neither result was statistically significant (P = 0.396 and 0.384, respectively; Fig. 1C). The lack of benefit for stage II patients was consistent with the QUASAR trial (2) and further supports the fact that the majority of these patients were cured by surgery alone. The lack of significant benefit from adjuvant chemotherapy for the stage III patients might reflect the relatively small number of patients in

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this group ($n = 47$ in the treatment arm and $n = 46$ in the observation arm).

**Correlations between individual markers**

As we have previously found that c-FLIP regulates cell viability and chemotherapy-induced apoptosis in colorectal cancer cells *in vitro* and *in vivo* by inhibiting DR5- and caspase 8–mediated apoptosis, the tissue microarrays were analyzed for the expression of DR5, TRAIL, c-FLIP, and caspase 8 as described in Materials and Methods. Representative images for each individual marker are shown in Fig. 2 for matched normal and tumor tissue. Within the adjacent normal tissue (Supplementary Table S2), there were positive correlations between the expression of c-FLIP and caspase 8 and DR5 (in all cases $P < 0.001$). Significant correlations also existed between DR5 and TRAIL ($P < 0.001$). In the tumor tissue, positive correlations existed between c-FLIP and both caspase 8 and DR5 expression (Supplementary Table S3; $P = 0.002$). In addition, a significant correlation existed between DR5 and caspase 8 ($P = 0.003$). Overall, the correlations between the apoptotic markers were stronger in the normal tissue than in the tumor tissue. This might reflect a greater degree of deregulated apoptosis in tumor cells and/or a greater degree of cellular heterogeneity.

**Marker expression and correlation with clinical outcome**

The overall mean expression scores of each individual marker in the normal and tumor tissues from the whole cohort are presented in Fig. 3A-D. In Supplementary Fig. S2, the expression of each marker between stage II and stage III colorectal cancer is compared. In addition, we determined the percentage of patients for whom the marker was overexpressed, underexpressed, or did not change between normal and tumor (shown in Fig. 3E). The results of these analyses are discussed below for each marker.

**TRAIL.** The expression of TRAIL was detected in 60% of the tumor samples and 78% of the matched normal samples. There were significantly higher expression scores in the normal tissue compared with the tumors ($P < 0.001$; Fig. 3A). When comparing matched normal and tumor tissue for each patient, 62% of patients had higher TRAIL expression in the normal tissue compared with 25% of patients who had higher TRAIL expression in their tumors (Fig. 3E). In univariate survival analysis of all patients (Table 2), inclusive of all stages and treatment arms, higher tumor TRAIL expression was significantly associated with decreased overall survival ($P = 0.026$) and a near significant trend towards impaired RFS ($P = 0.062$; Table 3). However, there were no significant correlations...
between tumor TRAIL expression and either RFS or overall survival when the cohort was divided by stage or by stage then treatment. TRAIL expression in the normal tissue had no effect on RFS or overall survival (Tables 2 and 3).

**DR5.** Expression of DR5 was detected in 96% of the tumor samples and in 80% of the adjacent normal tissue. Overall, there was significantly higher DR5 expression in the tumor tissues compared with matched normal tissues ($P < 0.001$; Fig. 3B), and this was reflected in the high percentage of patients (83%) with higher DR5 expression in their tumors compared with matched normal tissue (Fig. 3E). In univariate survival analysis inclusive of all patients, or on subgroup analysis divided by stage/treatment, there were no significant associations between DR5 expression and either RFS or overall survival in normal or tumor tissues (Tables 2 and 3).

**c-FLIP.** Expression of c-FLIP was detected in 99.5% of the tumor tissues and 99% of the normal tissues. Overall, there were significantly higher expression scores in the tumor tissue compared with the matched normal tissue ($P < 0.001$; Fig. 3C). Moreover, when the matched normal and tumor tissues for each patient were compared, it was found that the tumors of 84% of individuals overexpressed c-FLIP, whereas only 11% had reduced expression of c-FLIP (Fig. 3E). Notably, in univariate Cox regression analysis, inclusive of all patients, higher c-FLIP expression was associated with decreased RFS ($P = 0.015$), but failed to have a significant effect on overall survival.
High c-FLIP expression also had a significant adverse effect on RFS ($P = 0.026$) when the stage III individuals were considered alone.

**Caspase 8.** Expression of caspase 8 was cytoplasmic in nature and evident in 99.5% of tumor tissue and 90% of normal tissue. In agreement with previous studies (22, 23), there were significantly higher expression scores in the tumor tissue compared with the matched normal tissue (Fig. 3D, $P = 0.01$). Expression was markedly heterogeneous, both interindividually and also within cores from the same individual. Comparing matched normal and tumor samples, 56% of individuals displayed overexpression of caspase 8 in their tumors, 36% showed underexpression, and 8% had no change (Fig. 3E). Following univariate analysis of the entire cohort, and following subsequent subgroup analysis according to stage and

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**Fig. 3.** A–D, immunohistochemical expression signature scores were derived from the multiplication product of (staining intensity) $\times$ (extent) for each core. Significant differences in mean expression scores between matched normal and tumor samples were noted for TRAIL, DR5, caspase 8, and c-FLIP. $P$ values are two-tailed and represent paired $t$ test analysis. E, the percentage of patients displaying differences between matched normal and tumor samples; upregulation, downregulation, and no change for each individual marker is illustrated.
treatment, there was no association between the degree of caspase 8 expression and survival end points.

Assessment of biomarker-treatment interactions
In each of the individual biomarker expression analyses, a treatment interaction effect was sought to determine whether these potential biomarkers had predictive value in terms of the effect of chemotherapy treatment following surgical resection. None of these biomarkers significantly predicted response to chemotherapy treatment following surgical resection. However, on subdivision of stage III individuals into chemotherapy treatment versus observation alone groups, high tumor c-FLIP expression conveyed a nonsignificant trend for adverse outcome that was more significant in the chemotherapy treatment arm ($P = 0.07$ for the treatment arm and $P = 0.1$ in the observation arm). This finding has relevance to our previous studies, in which we have shown that c-FLIP is a critical inhibitor of chemotherapy-induced apoptosis in colorectal cancer cells in vitro and in vivo (17).

Multivariate analysis: exploratory predictive modeling
Using the backward log rank method of Cox regression analysis, whereby candidate variables are considered together and nonsignificant variables to the overall model are removed in a stepwise fashion, we attempted to create a predictive model for both RFS and overall survival for all patients in the cohort. We included age, stage, treatment, and all the normal and tumor expression marker scores in the model. With respect to RFS, two variables remained in the overall model after 12 elimination steps; these were disease stage (II versus III) and tumor c-FLIP expression ($P < 0.001$). When we examined overall survival, two variables were left in the model after 12 elimination steps: tumor TRAIL score and disease stage ($P < 0.001$). Therefore, in multivariate models, tumor c-FLIP and TRAIL expression hold adverse prognostic significance for RFS and overall survival, respectively.

Discussion
We have previously found that c-FLIP is a key regulator of colorectal cancer cell survival, both constitutively and in the context of chemotherapy and death ligand treatment, through its ability to regulate caspase 8 activation by the TRAIL death receptor, DR5 (15–17). In this study, we have assessed the expression and prognostic relevance of c-FLIP, caspase 8, DR5, and TRAIL in tissues from patients with stage II and III colorectal cancer using tissue microarrays constructed from matched normal and tumor tissue from patients with the disease. These patients ($n = 253$) were enrolled in a phase III trial of adjuvant 5-FU–based chemotherapy versus postoperative observation alone. There was a higher proportion of stage II (64%) than stage III patients (36%) in this trial. This was a reflection of the growing consensus during the time period of the trial that most stage III patients should be considered for adjuvant 5-FU–based chemotherapy. In fact, this change resulted in early closure of the trial with only 50% of the planned sample size reached. Consequently, the trial was not sufficiently powered to show that the use of adjuvant chemotherapy results in significantly increased survival for stage III patients.

In agreement with previous studies, the colorectal tumor tissues displayed marked upregulated expression of DR5 but lower TRAIL expression when compared with matched normal tissue (20, 25, 26). The relative overexpression of DR5 highlights the potential exploitation of TRAIL receptors as therapeutic targets in colorectal cancer. TRAIL receptor–targeted therapeutics are in clinical development (27), and we and others have shown that colorectal cancer cells are susceptible to TRAIL-induced apoptosis, both as a single agent and in combination with chemotherapy and targeted therapies (15, 28–31).

### Table 2. Effect of individual marker expression scores on overall survival for all patients ($N = 253$) as determined by Cox regression proportional analysis

<table>
<thead>
<tr>
<th>Marker</th>
<th>Regression coefficient estimate</th>
<th>SE</th>
<th>Significance</th>
<th>Risk ratio [Exp (B)]</th>
<th>95% CI for Exp (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>0.025</td>
<td>0.031</td>
<td>0.417</td>
<td>1.026</td>
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<tr>
<td></td>
<td>T</td>
<td>0.101</td>
<td>0.046</td>
<td>0.026*</td>
<td>1.107</td>
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<tr>
<td>TRAIL</td>
<td>N</td>
<td>0.078</td>
<td>0.066</td>
<td>0.237</td>
<td>1.081</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>-0.045</td>
<td>0.056</td>
<td>0.426</td>
<td>0.956</td>
</tr>
<tr>
<td>DR5</td>
<td>N</td>
<td>-0.003</td>
<td>0.037</td>
<td>0.944</td>
<td>0.997</td>
</tr>
<tr>
<td></td>
<td>T</td>
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<td>0.043</td>
<td>0.238</td>
<td>1.052</td>
</tr>
<tr>
<td>c-FLIP</td>
<td>N</td>
<td>-0.008</td>
<td>0.031</td>
<td>0.791</td>
<td>1.008</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>0.050</td>
<td>0.043</td>
<td>0.839</td>
<td>0.992</td>
</tr>
</tbody>
</table>

*$P < 0.05$. 

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However, we and others have also shown that the overexpression of c-FLIP in colorectal cancer cells limits the effectiveness of TRAIL receptor–targeted therapeutics and that downregulating c-FLIP synergistically enhances TRAIL-induced apoptosis (15, 29). Therefore, the overexpression of c-FLIP that we observed in the matched colorectal cancer tissues is likely to be a key resistance factor to TRAIL receptor–targeted therapeutics. Moreover, inhibiting c-FLIP expression or function is therefore likely to be important for maximizing the clinical activity of these agents.

In two previous studies in colorectal cancer, no prognostic association was found between TRAIL expression and clinical outcome (20, 21). However, in univariate analysis, we found that high tumor TRAIL expression in the entire cohort was associated with a significant 11% increase in the risk of death and had a near-significant trend to adversely affect RFS (P = 0.062). Tumor expression of Fas ligand has been linked to the so-called “Fas counterattack” in which tumor Fas ligand induces apoptosis of Fas-sensitive immune cells (32, 33), although this is somewhat controversial (34). It has also been proposed that tumor-expressed TRAIL could act in a similar way to suppress tumor-specific T-cell responses (35); this might provide an explanation for the adverse prognosis associated with TRAIL overexpression. In our hands, none of the other published antibodies and staining protocols gave the expected pattern of staining in these c-FLIP-overexpressing HCT116 colorectal cancer cell lines (data not shown). TRAIL-induced nuclear factor-kB induction is dependent on c-FLIP blocking caspase 8 activation and recruiting TNF receptor–associated factor-2 (39). Thus, in the presence of c-FLIP overexpression, TRAIL may activate nuclear factor-kB and thereby induce transcriptional upregulation of antiapoptotic genes rather than apoptosis. This might also partly explain the adverse clinical outcome in individuals overexpressing TRAIL. In agreement with previous studies (20, 21), we found that expression of DR5 was not prognostic.

We have previously found that caspase 8 is an important mediator of chemotherapy-induced apoptosis in colorectal cancer (17). Despite playing a pivotal role in death receptor-mediated apoptosis, to the best of our knowledge, the prognostic role of caspase 8 protein expression in colorectal cancer has not previously been reported. In the tumor tissue examined in this study, caspase 8 expression was significantly higher than in matched normal tissue. This is in agreement with another study in colorectal (22) and rectal cancer (23) and has important implications for the effectiveness of TRAIL receptor–targeted agents in colorectal cancer. Furthermore, these results suggest that caspase 8 has nonapoptotic functions that may contribute to tumorigenesis. Indeed, such nonapoptotic functions have been identified (13, 40, 41), and it seems that the loss of caspase 8 expression that is observed in certain cancers (for example, small cell lung cancer; ref. 42) is the exception rather than the rule. There was also a positive correlation between caspase 8 and c-FLIP tumor expression, however, caspase 8 expression was not found to be prognostic.

A variety of methods and antibodies have been evaluated for detecting c-FLIP by immunohistochemistry. We initially tested a range of c-FLIP antibodies including those used in other publications (18, 19), and of several antibodies tested, only the Santa Cruz c-FLIPS/L mouse IgG1 antibody (G-11) gave the immunohistochemical pattern of staining that we expected when tested on cell pellets derived from stable c-FLIPL and c-FLIPS overexpressing HCT116 colorectal cancer cell lines (data not shown). In our hands, none of the other published antibodies and protocols gave the expected pattern of staining in these c-FLIP overexpressing control samples. Despite the different antibodies used, our study agrees with a previous study that also found significantly higher expression of c-FLIP in colorectal tumor tissue compared with matched normal

### Table 3. Effect of individual marker expression scores on RFS for all patients (N = 253) as determined by Cox regression proportional analysis

<table>
<thead>
<tr>
<th>Marker</th>
<th>Regression coefficient estimate</th>
<th>SE</th>
<th>Significance</th>
<th>Risk ratio [Exp (B)]</th>
<th>95% CI for Exp (B)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
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<td>0.022</td>
<td>0.036</td>
<td>0.545</td>
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<tr>
<td></td>
<td>T</td>
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<td>0.053</td>
<td>0.062</td>
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<tr>
<td>DRS N</td>
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<td>0.076</td>
<td>0.484</td>
<td>1.054</td>
<td>0.909</td>
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<tr>
<td></td>
<td>T</td>
<td>−0.15</td>
<td>0.064</td>
<td>0.811</td>
<td>0.985</td>
</tr>
<tr>
<td>c-FLIP N</td>
<td>0.004</td>
<td>0.042</td>
<td>0.916</td>
<td>1.004</td>
<td>0.925</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>0.128</td>
<td>0.053</td>
<td>0.015*</td>
<td>1.136</td>
</tr>
<tr>
<td>Caspase 8 N</td>
<td>0.027</td>
<td>0.034</td>
<td>0.440</td>
<td>1.027</td>
<td>0.960</td>
</tr>
<tr>
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<td>T</td>
<td>−0.002</td>
<td>0.047</td>
<td>0.973</td>
<td>0.998</td>
</tr>
</tbody>
</table>

*P < 0.05.
tissue (43). Indeed, in their smaller series of 52 matched normal and tumor specimens, Ryu et al. found that a very similar percentage of tumors overexpressed c-FLIP (79% compared with 84% in our study). Based on our previous in vitro and in vivo studies (17), this overexpression of c-FLIP in colorectal tumors is a potentially important mechanism of resistance to chemotherapy. In support of this, high tumor c-FLIP expression conveyed a nonsignificant trend for adverse outcome in patients with stage III disease that was more significant in the chemotherapy treatment arm than in the observation arm.

A key finding of our study is that high c-FLIP expression was associated with a significant 14% increase in the relative risk of colorectal cancer recurrence for all patients combined and a 17% increase in the relative risk of recurrence when individuals with stage III disease were considered separately. Importantly, a multivariate predictive model for recurrence was found to be significant when stage and c-FLIP tumor scores were included. Two other studies have previously investigated the prognostic relevance of c-FLIP expression in colorectal cancer, and in addition to the different antibodies and scoring systems used, it is important to highlight the differences and similarities between those studies and our study. Ullenhag et al. analyzed c-FLIP expression in 396 stage I to IV colorectal cancer tumors by immunohistochemistry (no normal tissue analyzed) and defined expression as negative, weak, moderate, or strong as defined by intensity only (18). They found that “strong” c-FLIPi expression was associated with adverse disease-specific survival as determined by Kaplan-Meier analysis; c-FLIPi expression was not found to have a prognostic effect. However, only a very small percentage of tumors (19 of 396, <5%) were defined as having strong c-FLIPi expression; in the tumors that were classified as either weak (60%) or moderate (30%), c-FLIPi expression was not prognostic. In a smaller study (n = 90), c-FLIP expression was determined more quantitatively as the percentage of colorectal tumor cells with clear cytoplasmic positivity (19). Tumor immunoreactivity for c-FLIP was relatively low (69%) in this study compared with our study and other studies (18, 43). However, in agreement with our study, higher than median c-FLIP expression (defined as positivity in >10% of cancer cells) was associated with impaired survival in univariate and multivariate analyses. Overall, despite the differences in antibodies and scoring systems used, our work and that of others suggest that c-FLIP overexpression may be a potential adverse prognostic biomarker in colorectal cancer. However, there is clearly a need for more extensive studies and standardization of the protocols used to determine c-FLIP expression by immunohistochemistry.

In conclusion, we have found significant alterations in c-FLIP, caspase 8, DR5, and TRAIL expression between matched normal and tumor colorectal tissue, which might have important clinical implications for the effectiveness of chemotherapy and novel TRAIL-targeted therapies in this disease. Moreover, we have found that c-FLIP and TRAIL expression might convey significant prognostic information for stage II and III colorectal cancer, suggesting that this signaling pathway is relevant for the pathogenesis of this disease. Additional studies are warranted in larger patient cohorts to confirm the prognostic significance of c-FLIP and TRAIL in colorectal cancer. Preferably, these studies should be conducted in a blinded, prospective manner.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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