Regulation of TRAIL receptor expression by β-catenin in colorectal tumours

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Tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL) is being investigated as a targeted cancer therapeutic and the expression of its pro-apoptotic receptors, DR4 and DR5, increases during colorectal carcinogenesis. This study investigated the role of β-catenin in the regulation of these receptors. In human colorectal adenoma and carcinoma cell lines, downregulation of β-catenin resulted in lower total DR4 and DR5 protein levels. Similarly, membrane expression of DR4 and DR5 was reduced after downregulation of β-catenin in colon carcinoma cells, whereas induction of β-catenin in HeLa cells led to increased cell membrane expression of DR4 and DR5. Downregulation of β-catenin decreased the recombinant human TRAIL sensitivity. Activation of the transcription factor T-cell factor-4 (TCF-4) is an important function of β-catenin. Dominant-negative TCF-4 overexpression, however, did not significantly affect TRAIL receptor expression or recombinant human TRAIL sensitivity. Human colorectal adenomas (N = 158) with aberrant (cytoplasmic and nuclear) β-catenin expression had a higher percentage of immunohistochemically DR4 and DR5 staining per tumour (mean: 73 and 88%, respectively) than those with membranous β-catenin staining only (mean: 50 and 70%, respectively, P < 0.01 for both). Furthermore, aberrant β-catenin staining co-localized with DR4 and DR5 expression in 93% of adenomas. In 53 human colorectal carcinomas, aberrant β-catenin expression was present in most cases and DR4/5 expression was largely homogenous. Similarly, in adenomas from APCmin mice, cytoplasmic β-catenin staining co-localized with staining for the murine TRAIL death receptor. In conclusion, the gradual increase in TRAIL receptor expression during colorectal carcinogenesis is at least partially mediated through increased β-catenin expression, independently of TCF-4-signalling.

Introduction

Tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL) is a cytokine that can induce apoptosis by binding to its pro-apoptotic receptors DR4 and/or DR5 (1). The immunohistochemical expression of these receptors increases during the adenoma–carcinoma sequence and may confer a growth advantage (2–5).

Recombinant human TRAIL (rhTRAIL) can induce apoptosis in human colon adenoma and carcinoma cell lines and human colorectal adenomas (6,7). In view of this, and its minimal toxicity for normal cells in vitro and in vivo, rhTRAIL and other TRAIL agonists are exciting potential drugs for the treatment and prevention of colorectal carcinoma (8). They are currently in early phase clinical trials and showing favourable toxicity profiles and indication of clinical activity (9). Recent reports suggest that a subset of cancer cell lines, with receptor-proximal apoptosis defects, show increased proliferation in response to TRAIL treatment and that oncogenic K-Ras can convert death receptors (DRs) into invasion-inducing receptors (10,11). It is therefore essential to understand the regulation and functionality of the TRAIL receptors targeted in the selection of patients who may benefit from TRAIL treatment.

The increase in TRAIL receptor expression during colorectal carcinogenesis is currently unexplained but may be related to Wnt pathway activation. Increased TRAIL receptor expression and Wnt pathway activation are both associated with worse survival in patients with colorectal cancer (4,12). The initiation and progression of the majority of colorectal carcinomas involves Wnt pathway activation. The Wnt pathway is activated due to adenomatous polyposis coli (APC) or β-catenin (CTNNB1) mutations leading to inadequate degradation of β-catenin in the cytoplasm. This results in nuclear accumulation of β-catenin and transcriptional activation of T-cell factors (TCFs), which regulate genes involved in cell proliferation and apoptosis (13). The accumulation of β-catenin in both cytoplasm and nucleus, as well as the overexpression of TCF-4-target gene products, is seen in colorectal adenomas and to a greater extent in colorectal carcinomas (14,15).

The hypothesis of this study is that β-catenin accumulation is involved in the upregulation of the pro-apoptotic TRAIL receptors and that enhanced β-catenin levels promote TRAIL sensitivity. We investigated whether increased expression of β-catenin and subsequent TCF-4 activation are involved in the increase of DR4/5 expression during colorectal carcinogenesis. Firstly, TRAIL receptor expression and rhTRAIL sensitivity were investigated in human colon adenoma and carcinoma cell lines in which β-catenin and/or TCF-4 signalling could be downregulated. Secondly, TRAIL receptor expression was investigated after induction of β-catenin in cell lines with low intrinsic levels. Finally, immunohistochemical staining of DR4/5 in relation to the expression of β-catenin was investigated in human and mouse gastrointestinal tumours.

Materials and methods

Cell lines

LS174T transfectant human colon carcinoma cell lines (LS174T-BcatRNAi, LS174T-dnTCF-4 and LS174T) containing doxycyclin-inducible expression plasmids were used (16). LS174T cells carry a mutant, oncogenic β-catenin allele and actively transcribe TCF reporter constructs. The ‘LS174T-BcatRNAi’ has an inducible β-catenin-short-hairpin RNA vector, which allows the rapid production of small interfering RNAs (siRNAs) against β-catenin on doxycyclin induction. The ‘LS174T-dnTCF-4’ has a doxycyclin-inducible, FLAG-tagged, plasmid encoding N-terminally truncated versions of TCF-4. These dominant-negative TCF-4 (dnTCF-4) proteins do not bind β-catenin and act as potent inhibitors of TCF-4 signalling. The ‘LS174T’ is stably transfected with the doxycyclin responsive promoter and an empty vector as a control (16). Using this cell line model, it is possible to downregulate Wnt pathway activity at the β-catenin or the TCF-4 level. The cell lines were cultured in RPMI enriched with 5% fetal calf serum at 37°C in a humidified atmosphere with 5% CO2. The cells were harvested by treatment with trypsin. The cells were put under selection with blasticidin 10 μg/ml and zeocin 500 μg/ml for 1 week each month.

V ACO-235 and V ACO-330 are human adenoma cell lines, with receptor-proximal apoptosis defects, show increased proliferation in response to TRAIL treatment and that oncogenic K-Ras can convert death receptors (DRs) into invasion-inducing receptors (10,11). It is therefore essential to understand the regulation and functionality of the TRAIL receptors targeted in the selection of patients who may benefit from TRAIL treatment.

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To induce dnTCF-4 expression and β-catenin RNAi production, doxycyclin was added at a final concentration of 1 μg/ml. HeLa cells (human cervical carcinoma cell line), which have low intrinsic levels of β-catenin, were cultured in RPMI enriched with 10% fetal calf serum in a humidified atmosphere with 5% CO2. The cells were harvested by treatment with trypsin. The cells were put under selection with blasticidin 10 μg/ml and zeocin 500 μg/ml for 1 week each month.

Abbreviations: APC, adenomatous polyposis coli; cDNA, complementary DNA; DR, death receptor; rh, recombinant human TRAIL; RT–PCR, reverse transcription–PCR; siRNA, small interfering RNA; TCF, T-cell factor; TRAIL, TNF-related apoptosis-inducing ligand.
β-catenin and TRAIL receptor expression regulation

β-catenin RNA-interference in adenoma cell lines

siRNAs specific for human β-catenin were synthesized by Eurogentec (Seraing, Belgium): 5'-GUG GUG GGU AUA GAG GCC C99-3' (sense) and 5'-GAG CCU CTA TAC CAC CCA C99-3' (antisense). Double-stranded RNA molecules [5'-CUU ACG CUG AGU AUU CGG AdTdT-3' (sense) and 5'-GUC AAG UAC UCA GCG UAA GdTdT-3' (antisense)] specific for the luciferase (Luc) gene served as controls. Flow cytometric analysis of VACO-235 and VACO-330 cells transfected with fluorescein-5-isothiocyanate-labelleled oligonucleotides revealed a transfection efficiency of 85 and 65%, respectively. VACO-235 and VACO-330 cells were transfected in six-well plates with 10 ml of 20 μM siRNA duplexes using Oligofectamine reagent according to the manufacturer’s instructions (Invitrogen BV, Breda, the Netherlands). After 48 h, cells were harvested for protein isolation or fluorescence activated cell sorting analysis.

Induction of β-catenin in HeLa cells

β-catenin expression was induced in HeLa cells by incubation with 30 mM of lithium chloride. Lithium causes stabilization and accumulation of β-catenin through inhibition of glycogen synthase kinase-3β, which leads to reduced phosphorylation and degradation of β-catenin (18). Control cells were incubated with 30 mM of sodium chloride. After 24 h, cells were harvested for protein isolation or fluorescence activated cell sorting analysis.

Membrane expression of TRAIL receptors

The Ls174 transfectant cell lines were induced with doxycyclin for 48 h prior to analysis. VACO-235 and VACO-330 cells were transfected with siRNA for β-catenin and DR4, as described above. Cells were harvested 2 days later (16) and resuspended in medium (phosphate-buffered saline, 2% fetal calf serum, 1% sodium azide). TRAIL receptor membrane expression was analysed using a flow cytometer (Epicus Elite, Coulter-Electronics, Hialeah, FL) and cells were stained as described earlier (7). The following antibodies were used: huTRAILR1-M271 for DR4 and huTRAILR2-M413 for DR5 (a gift from Amgen, Seattle, WA). Membrane receptor expression is shown as mean fluorescence intensity of all analysed cells. All experiments were performed in triplicate.

The Ls174 transfectant cells were induced with doxycyclin for 48 h or treated with cycloheximide 2 μg/ml (Sigma–Aldrich Chemie BV, Zwijndrecht, The Netherlands) for 24 h prior to analysis. The HeLa cells were induced with lithium chloride for 24 h prior to analysis. Cells were harvested as described above. Western blot analysis was carried out as described previously (7). The following primary antibodies were used: a mouse monoclonal specific for β-catenin (1:2000, Transduction Laboratories), a goat polyclonal IgG specific for DR4 (1:500; clone C-20, Santa Cruz Biotechnology), a rabbit polyclonal IgG specific for DR5 (1:500; Oncogene Research), a rabbit polyclonal specific for c-myc (1:500; clone n-262, Santa Cruz), a mouse monoclonal specific for FLAG (1:1000, Sigma–Aldrich) and a mouse monoclonal specific for actin (1:10 000, ICN Biomedicals, Zoetermeer, The Netherlands). The secondary antibodies were labelled with horse radish peroxidase (all from DAKO, Heverlee, Belgium) and chemiluminescence was detected using the ECL-chemiluminescence kit or with the Lumi-Light Plus Western blotting kit (DAKO, Heverlee, Belgium) and chemiluminescence was detected using the ECL-chemiluminescence kit or with the Lumi-Light Plus Western blotting kit (DAKO, Heverlee, Belgium).

Real-time reverse transcription–PCR

Total RNA was isolated by guanidine isothiocyanate-phenol-chloroform extraction using TRIzol (Invitrogen) according to the manufacturer’s protocol. Total RNA was purified with the RNeasy mini kit (Qiagen, Leusden, The Netherlands) according to the manufacturer’s instructions. Trace amounts of DNA contamination were removed by on-column DNAse I digestion followed by analysis of tissues was executed using rat-anti-mouse DR5 (sc-73913, dilution 1:50, Santa Cruz Biotechnology, Santa Cruz, CA) and mouse-anti-β-catenin (610154, dilution 1:100, Becton Dickinson, Breda, the Netherlands).

Immunohistochemistry in human colorectal tumours

Immunohistochemistry for β-catenin, DR4 and DR5 was carried out in patient-derived, untreated, colorectal tumours. Paraffin-embedded colorectal adenomas and carcinomas were retrieved from the pathology department of the University Medical Center Groningen. Immunohistochemical staining was carried out as described previously (3). The variations in staining intensity of DR4/5 positive cells were very small, therefore immunohistochemical expression was scored as positive or negative. DR4/5 expression for each tumour was estimated semi-quantitatively as the percentage of positive cells within the tumour, where tumours with <10% positive cells were considered negative.

For β-catenin, antigen retrieval was performed by microwave treatment for 8 min at 700W in 0.1 M citrate buffer (pH 6.0). A monoclonal antibody for β-catenin (1:1000, Transduction Laboratories, Lexington, KY) was applied for 60 min. This was followed by appropriate secondary and tertiary antibodies and peroxidase activity was visualized with diaminobenzidine. Colon carcinoma and normal colon epithelium were used as positive controls. In colon carcinoma, β-catenin staining is localized in the cytoplasm and nucleus, whereas in normal colon epithelium β-catenin staining is membranous. The pattern of staining for β-catenin was recorded as membranous, cytoplasmic or nuclear and the percentage of cells with nuclear or cytoplasmic staining was estimated, β-catenin staining was defined as nuclear when at least 10% of the epithelial cells had nuclear staining.

To further investigate the relationship between β-catenin and DR4/DR5 staining in colorectal adenomas, co-localization was investigated in serial slices of the 13 smallest adenomas. Larger adenomas would have to be photographed in multiple sections making analysis more difficult. Each adenoma was photographed at a ×25 magnification. Image-pro plus (MediaCybernetics, Silver Spring, MD) was used to synchronize the orientation of the adenomas and using a grid each adenoma was divided into equal squares, the number (mean: 180, range: 84–490) depending on the size of the adenoma. For DR4/5 staining each square containing epithelial cells was evaluated as positive or negative. For β-catenin staining, squares with cytoplasmic and/or nuclear staining were considered positive and squares with membranous staining were considered negative.

Immunohistochemistry in adenomas of APC<sup>min</sup> mice

Immunohistochemistry was performed on the intestines of APC<sup>min</sup> mice used in an 18F-FDG-PET imaging study (21). Experimental protocols were approved by the Institutional Animal Care Committee. Heterozygous male C57BL/6JAnJ (N45) mice (age, 5 weeks) were obtained from Jackson Laboratories. Animals were housed in groups (4–5 mice) in a temperature-controlled room with a 12-h light–dark cycle and ad libitum food and water. Mice were fed standard chow. All animals received fresh food weekly. Animals were killed at 8 weeks by cervical dislocation. The entire intestine from the pylorus to the rectum was removed and placed in ice-cold phosphate-buffered saline, pH 7.0. After macroscopic examination, the intestines were paraffin embedded. Histological analysis was performed for the generation of the real-time reverse transcription–PCR. The expression of tissues was executed using rat-anti-mouse DR5 (sc-73913, dilution 1:50, Santa Cruz Biotechnology, Santa Cruz, CA) and mouse-anti-β-catenin (610154, dilution 1:100, Becton Dickinson, Breda, the Netherlands). 

β-catenin and TRAIL receptor expression regulation.
Statistical analysis

SPSS for Windows software (SPSS, Chicago, IL) was used. P values < 0.05 were considered significant. Comparisons between groups regarding histopathological characteristics were evaluated using the Mann–Whitney U test for two independent samples not normally distributed and linear regression analysis. To determine co-localization between stainings the kappa coefficient was determined. The McNemar test was used to investigate discordance between staining results. The Student’s t-test was used to determine differences between cell lines exposed to various conditions.

Results

Downregulation of β-catenin leads to reduced DR4/5 expression and reduced TRAIL sensitivity

The effect of downregulation of β-catenin on DR4 and DR5 expression was investigated in human colon carcinoma and adenoma cell line models. All cell lines used exhibited basic membranous expression of DR4 and DR5. Long-term downregulation (>72 h) of β-catenin led to growth inhibition in all cell lines used; however, in the relatively short time period of our experiments, this effect was not present and therefore it did not influence the results. Downregulation of β-catenin in the human LS174T-CBcatRNa1 carcinoma cell line led to a time-dependent reduction in the protein expression of the Wnt target gene c-myc (Figure 1A). In the adenoma cell lines, β-catenin downregulation also resulted in a decreased c-myc expression (Figure 1B). This demonstrates functional inhibition of the Wnt pathway, in both carcinoma and adenoma cell line models. Downregulation of β-catenin was accompanied by reduced protein expression of both DR4 in all cases and DR5 in the carcinoma cell lines and the VACO-235 adenoma cell line (Figure 1A and B). These reductions in DR4 and DR5 expression were comparable with those achieved by treating the cells with the protein synthesis inhibitor cycloheximide for 24h (data not shown). Membrane expression of DR4 and DR5 was also reduced after downregulation of β-catenin in the colon carcinoma cell line model (29 and 39% reduction, respectively, P < 0.05; Figure 1C). RNA levels of DR4 and DR5 remained unaltered (data not shown). In HeLa cells, the protein expression of β-catenin was increased by using lithium chloride, a glycogen synthase kinase-3β inhibitor (Figure 1D). Increased β-catenin expression led to increased membrane expression of both DR4 and DR5 (116 and 287% increase, respectively; P < 0.05 for both (Figure 1E and F). RNA levels of DR4 and DR5 remained unaltered (data not shown).

Downregulation of β-catenin in the LS174T colon carcinoma cell line model reduced rhTRAIL-induced apoptosis (P < 0.05, Figure 2A). This effect was seen at both rhTRAIL concentrations used. This indicates that the reduction in DR4/5 expression is associated with a reduced sensitivity to rhTRAIL. None of the reported effects were seen in the control cell line, LS174T, indicating that it is not an effect induced by the addition of doxycyclin itself.

β-catenin can bind to the transcription factor TCF-4 in the nucleus and so activate transcription of TCF-4 target genes. The LS174T-dnTCF-4 cell line was used to investigate whether the effect of β-catenin levels on DR4/5 expression is TCF-4 mediated. Doxycyclin-induced expression of FLAG-tagged dnTCF-4 protein was shown by staining for the FLAG protein and the dominant-negative effect of dnTCF-4 expression was demonstrated by the reduced protein expression of the TCF-4-target gene c-myc (Figure 1A). Expression of dnTCF-4 did not influence protein and membrane levels of DR4 and DR5 nor did it influence rhTRAIL sensitivity at the higher dose (Figures 1 and 2B). At the lower dose rhTRAIL sensitivity was slightly decreased (Figure 2B). Taken together, these results suggest that the regulation of DR4 and DR5 expression by β-catenin is not solely mediated through TCF-4 in this model.

β-catenin expression in colon adenomas is associated with DR4 and DR5 expression

To investigate the role of β-catenin in the regulation of DR4/5 expression in vivo, expression patterns of β-catenin, DR4 and DR5 were investigated in patient-derived colorectal tumours using immunohistochemistry. The characteristics of this group of tumours are shown in Table I. Cytoplasmic staining and cytoplasmic plus nuclear staining of β-catenin were more frequently observed in carcinomas (100 and 49% of the tumours, respectively) than in adenomas (90 and 15%, respectively) indicating increasing activation of the Wnt pathway during the adenoma–carcinoma sequence as expected. The mean percentage of DR4 and DR5 positive cells per tumour was higher in carcinomas than in adenomas (89 versus 51%, P < 0.001 and 94 versus 83%, P < 0.001, respectively). In adjacent normal tissue expression of DR4 and DR5 was low and β-catenin expression was predominantly membranous.

Subsequently, the results of the β-catenin, DR4 and DR5 stainings were combined. More intense immunohistochemical DR4 staining was observed in colorectal adenomas with nuclear β-catenin staining than in those without (Figure 3). In the multivariate analysis, the association between nuclear β-catenin staining and DR4 staining (P = 0.001) in adenomas was shown to be independent of size, degree of dysplasia and growth type of the adenoma and age of the patients. More intense DR5 staining was observed in colorectal adenomas with either cytoplasmic or cytoplasmic and nuclear β-catenin staining than in those with membranous β-catenin staining only. In human carcinomas, cytoplasmic β-catenin staining was largely homogenous with foci of nuclear staining and all tumours were almost entirely DR4 and DR5 positive. It was therefore not possible to analyse the association between β-catenin and DR4/5 staining in carcinomas separately.

β-catenin co-localizes with DR4 and DR5

To further examine this relationship, the co-localization of β-catenin and DR4 and DR5 expression within small subsections of the adenomas was investigated in serial slides of the smallest adenomas. Subsections with increased intensity of cytoplasmic β-catenin staining compared with surrounding tissue and/or nuclear staining co-localized with subsections of increased DR4 and DR5 staining in 12/13 adenomas (Figure 4A-D, Table II). This indicates an association between β-catenin and both DR4 and DR5 expressions. The majority (78%) of grid sections where DR4 and DR5 co-localized showed increased β-catenin staining, suggesting a role for β-catenin as a common regulator of both receptors. The McNemar test showed that there is discordance between staining due to the fact that positive β-catenin staining was more common than positive DR4 or DR5 staining and therefore the expression patterns of these stainings can never be identical. Consequently, maximum kappa values will always be smaller than one for this data set. To further investigate this relationship, we analysed β-catenin and murine TRAIL DR expression in adenomas from APC<sup>min</sup> mice. In these adenomas, β-catenin and murine TRAIL DR were also found to co-localize (Figure 4E and F). Staining intensities were independent of size of the polyps and age of the mice. In summary, the immunohistochemistry results show that increased cytoplasmic and/or nuclear staining of β-catenin is associated with increased expression of the human TRAIL receptors DR4 and DR5 and the mouse TRAIL DR.

Discussion

This study shows that in human colorectal adenomas and carcinomas and in adenomas from APC<sup>min</sup> mice, aberrant β-catenin staining is associated with, and co-localizes with, increased staining of pro-apoptotic TRAIL DRs. We demonstrate, using functional experiments in colon adenoma and carcinoma cell line models, that downregulation of β-catenin leads to lower membranous expression of DR4 and DR5 and a reduced TRAIL sensitivity. In the reciprocal experiment, induction of β-catenin expression leads to increased membrane expression of DR4 and DR5.

The best-known role of β-catenin in colon carcinoma is activation of Wnt signalling. Activation of the Wnt pathway, as determined through expression of TCF-4 target genes, is seen in early adenomas and increases during colorectal carcinogenesis (14). The influence of β-catenin on DR4 and DR5 expression suggests that these receptors
β-catenin and TRAIL receptor expression regulation

Fig. 1. LS174T, LS174T-βcatRNAi and the LS174T-dnTCF-4 cells were treated with doxycyclin for 0, 24 and 48 h to induce expression of target sequences. (A) Western blot analysis of β-catenin, c-myc, FLAG, DR4 and DR5 expression in LS174T cells. One representative of at least three different experiments is shown. The dnTCF protein has a FLAG-tag; therefore, FLAG expression demonstrates expression of dnTCF protein. Actin is shown as loading control. (B) Western blot analysis of β-catenin, c-myc, DR4 and DR5 expression in VACO-235 and VACO-330 cell lines. VACO-235 and VACO-330 cells were exposed to oligofectamine alone (cont) or transfected with siRNA duplexes directed against luciferase (luc) as control siRNA or against β-catenin (βcat) for 48 h. One representative of at least three different experiments is shown. Actin is shown as loading control. (C) Membrane expression of the TRAIL receptors DR4 and DR5 in the LS174T, LS174T-βcatRNAi and the LS174T-dnTCF-4 as determined by flow cytometry. Values are expressed as the percentage change in the mean fluorescence intensity after 48-h induction with doxycyclin and are mean ± standard deviation of at least three independent experiments. *The membrane expression of DR4 and DR5 was lower after 48 h of doxycycline induction in the βcatRNAi cells compared with the control cells (P < 0.05). (D) Western blot analysis of β-catenin expression in HeLa cells after incubation with increasing concentrations of lithium chloride or sodium chloride for 6 h. Actin is shown as a loading control. (E) Representative histograms of DR4 and DR5 expression levels measured by flow cytometry after 24 h of treatment with sodium chloride (control) or lithium chloride. Increased receptor expression after lithium chloride treatment was detected as an increased fluorescence intensity of the whole cell population and resulted in a peak shift to the right. (F) Membrane expression of the TRAIL receptors DR4 and DR5 in HeLa cells after 24 h of lithium chloride treatment as determined by flow cytometry. Values are expressed as the percentage difference in the mean fluorescence intensity between lithium-chloride-treated cells and control cells treated with sodium chloride and are mean standard deviation of at least three independent experiments. *The membrane expression of DR4 and DR5 was higher after 24 h of lithium chloride treatment compared with the control cells (P < 0.05).
could be direct or indirect targets of TCF-4 signalling. There are, however, no TCF-4 consensus binding sequences (5′-CCTTTGATC-3′) in the promoter regions of DR4 and DR5 and microarray studies comparing gene expression patterns between cells with active and inactive Wnt signalling did not identify the TRAIL receptors DR4 and DR5 as TCF-4 target genes (22–25). This is in line with our results indicating that inhibition of TCF-4 signalling does not influence DR4/5 expression levels.

The TCF-4 target gene c-myc has been associated with DR5 expression and TRAIL sensitivity. Dominant-negative c-myc reduced both DR5 expression and TRAIL sensitivity (26). Downregulation of glycogen synthase kinase-3β using siRNA resulted in increased DR5 expression and increased sensitivity to DR5 antibody treatment in some but not all cell lines tested (27). In contrast, others showed that c-myc expression reduced expression of cellular FLICE inhibitory protein (c-FLIP), an inhibitor of TRAIL-induced apoptosis, thus increasing TRAIL sensitivity without affecting TRAIL receptor levels (28). c-myc expression levels are influenced through many other mechanisms besides aberrant Wnt signalling (26, 29, 30). It seems that the effect of Wnt-signalling inhibition of c-myc is not sufficient to influence TRAIL receptor expression in our colon carcinoma model.

Interestingly, osteoprotegerin, which is a TRAIL decoy receptor and neutralizes its function, is regulated by β-catenin through the TCF transcription factor. In contrast to the DR4 and DR5 promoters, the osteoprotegerin promoter has three potential TCF-binding sites (31,32).

The lack of influence of β-catenin on RNA levels of the TRAIL receptors indicates that the effects are likely to be post-transcriptional, with influence on processes such as cell adhesion or protein stability (33). β-catenin plays an important role in cell adhesion and is found at the adherens junctions in a complex also including E-cadherin. Interestingly, both the E-cadherin–catenin complex and the pro-apoptotic TRAIL receptors have been reported to be located

### Table 1. Patient and tumour characteristics

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<tr>
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</table>

![Fig. 2.](https://example.com) Downregulation of β-catenin results in reduced TRAIL sensitivity. (B) Dominant-negative TCF expression did not result in reduced TRAIL sensitivity (1 μg/ml). At the lower rhTRAIL dose (0.1 μg/ml), there was a non-significant reduction in TRAIL sensitivity after dominant-negative TCF expression. dnTCF-Ls174T, Ls174T-βcatRNAi and the Ls174T-dnTCF-4 control and rhTRAIL 0.1 or 1 μg/ml, with and without 48 h of induction. Apoptosis was determined by acridine orange staining and is expressed as a percentage of the total number of cells counted. *The percentage of apoptosis in LS174T-βcatRNAi cells was lower after induction of the RNAi expression constructs (P < 0.05).

![Fig. 3.](https://example.com) Percentage of DR4 and DR5 positive cells in human colorectal adenomas as determined in adenomas with membranous staining only (M), with cytoplasmic staining only (C) and cytoplasmic and nuclear staining (CN) of β-catenin. Values are expressed as individual values (dots) and the mean staining percentages (lines).
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in so-called lipid rafts at the cell membrane (34,35). Loss of membranous β-catenin, as seen in tumor cells with cytoplasmic and nuclear staining, may facilitate stable expression of the pro-apoptotic DRs. β-catenin can also interact with the growth factor receptors c-Met and c-erbB2, the mucin MUC1 and the FOXO transcription factors, which could directly or indirectly influence expression of DR4 and DR5 (36–39).

The signal recognition particle has previously been shown to play an essential role in trafficking DR4 to the cell surface. DNA damage, oncogenic mutations leading to Ras activation and expression of

Fig. 4. β-catenin (A, B) and DR4 (C, D) stained tissue sections of a human adenoma showing co-localization of staining, before (A, C) and after (B, D) accentuation of contrast. Original magnification ×25. β-catenin (E) and mouse TRAIL DR (F) stained tissue sections of distal small intestine of an ApcMin mouse, 8 weeks of age showing co-localization of staining. Original magnification ×100, insert ×400.
wild-type p53 protein have also previously been described as regulators of DR4/5 expression (40–43). Our results indicate that β-catenin can be added to this list. In our cell line model, short-term suppression of β-catenin resulted in ~30% reductions in DR4/5 levels at the cell membrane and in the reciprocal experiment induction of β-catenin resulted in a >100% increase in DR4/5 membrane expression. The association between β-catenin and DR4/5 levels was even more pronounced in vivo in human colorectal adenomas, where a clear co-localization of β-catenin positive cells and DR4/5 positive cells was observed. This may be related to the longer time frame during which β-catenin is differentially expressed in colorectal adenomas in vivo compared with the in vitro model. Short-term downregulation of β-catenin may not be sufficient to completely reverse this process.

The association between β-catenin expression, TRAIL receptor expression and TRAIL sensitivity in this study underlines the fact that understanding TRAIL receptor regulation in both malignant and normal tissue is essential to predict the efficacy and toxicity of TRAIL receptor agonists. Non-steroid anti-inflammatory drugs have been shown to sensitize colon cancer cells to TRAIL-induced apoptosis in a TCF-4-dependent manner (44–46). Non-steroid anti-inflammatory drugs and other drugs can also reduce endogenous β-catenin levels (47). Therefore, trials of drug combinations including TRAIL should not only take into account TCF-4-dependent effects but also study pre- and post-treatment membrane expression levels of DR4/5 and β-catenin expression levels to identify counteractive effects of specific combinations. This is important since rhTRAIL and agonistic TRAIL receptor antibodies have reached phase I/II clinical trials and are being combined with other therapeutic agents in subsequent phases of clinical testing (48,49). In conclusion, we have demonstrated that the gradual increase in TRAIL receptor expression during colorectal carcinogenesis is at least partially mediated through increased β-catenin expression.

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**Conflict of Interest Statement:** None declared.

### References


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**Table II.** Co-localization of β-catenin, DR4 and DR5 staining as determined in all equal grid sections containing colon epithelium in 13 adenomas

<table>
<thead>
<tr>
<th>Protein 1</th>
<th>Protein 2</th>
<th>Overlap</th>
<th>Kappa value* (range)</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>β-catenin</td>
<td>DR4</td>
<td>1126</td>
<td>809</td>
<td>531</td>
</tr>
<tr>
<td>β-catenin</td>
<td>DR5</td>
<td>1126</td>
<td>753</td>
<td>474</td>
</tr>
<tr>
<td>DR4</td>
<td>DR5</td>
<td>809</td>
<td>753</td>
<td>429</td>
</tr>
</tbody>
</table>

*a The number of positive grid sections in each category is shown. Total grid sections = 4052.

*b Overlap is the number of grid sections with positive staining for protein 1 and protein 2.

Kappa = 0 if overlap is random and Kappa = 1 if both stainings are identical.
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