Chapter 5

Expression of TRAIL receptors DR4 and DR5 in sporadic and hereditary colorectal tumours: potential targets for apoptosis induction.

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Abstract

Background: Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and antibodies against TRAIL receptors death receptor 4 (DR4) and death receptor 5 (DR5) are under investigation for cancer therapy. To study potential application of these agents, the expression of DR4 and DR5 were studied in colorectal adenomas and carcinomas from patients with sporadic and hereditary disease. The role of the BAX gene, frequently mutated in tumours with high frequency microsatellite instability (MSI-H) and playing a role in sensitivity to TRAIL, was also studied.

Methods: Adenomas and carcinomas from patients with sporadic disease (n = 74 and 56 respectively), familial adenomatous polyposis (FAP, n = 41 and 4 respectively) and hereditary nonpolyposis colorectal cancer (HNPCC, n = 50 and 21 respectively) were studied by immunohistochemistry. MSI-H carcinomas (n = 42, of which 27 sporadic and 15 HNPCC) were analysed for apoptotic activity, assessed by M30 immunoreactivity, and BAX mutations.

Results: Most adenomas from all three patient groups expressed DR4 and DR5. Most carcinomas expressed DR4, except for 6 cases, all with mucinous histology. All carcinomas, including mucinous carcinomas, showed DR5 expression. BAX mutations were found in 6/42 MSI-H cancers with similar apoptotic indices and expression of DR4, DR5 and TRAIL in BAX mutant and wild-type cases.

Conclusion: Since most sporadic and hereditary colorectal neoplasms express DR4 and DR5, targeting of these receptors may be a potential prevention or treatment strategy. No evidence was found supporting the concept of BAX inactivation as a critical mechanism to evade TRAIL-receptor-mediated apoptosis in vivo.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality in the western world. The shortcomings of current treatment modalities for CRC call for novel strategies to treat or prevent the disease. Attention focuses on early detection of CRC or its precursor lesion, the adenoma, for example by endoscopic screening. An alternative approach to prevent the development of adenomas or carcinomas is the use of chemopreventive agents, especially in high-risk patients. Two major entities are known to carry a highly increased risk of developing CRC: familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) [1]. FAP, caused by a germline mutation in the APC gene is clinically characterised by numerous adenomatous polyps in the colon. HNPCC is caused by a germline mutation in one of the DNA mismatch repair (MMR) genes, in particular hMSH2, hMLH1 and hMSH6. As a consequence of defective DNA mismatch repair, tumours from HNPCC patients are characterised by length alterations in repetitive sequences distributed throughout the genome, so-called microsatellite instability (MSI) [2]. The MSI phenotype is also found in 10-15 % of sporadic CRC cases, as a result of hypermethylation of the hMLH1 gene promoter region [3].

In FAP patients, chemoprevention using nonsteroidal anti-inflammatory drugs (NSAIDs) reduced adenoma size and number in several studies [4]. However, complete regression of

adenomas in FAP patients is unusual, CRC can develop during NSAID treatment and long-term NSAID use is associated with side effects [5]. Chemoprevention studies in HNPCC patients are underway, however, it has been suggested that NSAIDs may be less effective in the setting of MSI [6]. The development of other agents is therefore needed.

TNF-related apoptosis inducing ligand (TRAIL) is a type II transmembrane protein which induces apoptosis in a variety of tumour cell lines but not in normal cells [7]. Four membrane-bound receptors for TRAIL have been identified: death receptor 4 (DR4), death receptor 5 (DR5), decoy receptor 1 (DcR1) and decoy receptor 2 (DcR2) [7]. TRAIL induces apoptosis by binding to DR4 or DR5, whereas DcR1 and DcR2 do not transduce apoptotic signals. Apoptosis induction through pro-apoptotic death receptors by recombinant human (rh) TRAIL or agonistic antibodies against these receptors is considered a promising approach for cancer therapy [8,9]. Expression patterns of DR4 and DR5 have been described in normal colon, sporadic adenomas and sporadic carcinomas [10,11]. The majority of tumours show DR4 and DR5 expression, with stronger intensity in neoplastic cells compared to normal tissue [10,11], suggesting preferential susceptibility of these cells to TRAIL-receptor mediated apoptosis. Whether the same expression patterns apply to hereditary cases is currently unknown.

Recently, BAX, a pro-apoptotic member of the Bcl-2 family, has been shown to play a role in sensitivity to TRAIL-mediated apoptosis in vitro [12-15]. Since up to half of colorectal tumours with high frequency MSI (MSI-H) contain frameshift mutations in a polyG tract of the BAX gene [2], this could potentially limit the use of TRAIL or agonistic antibodies in MSI-H tumours.

The aims of this study were twofold. First, DR4 and DR5 expression was investigated in colorectal tumours from patients with sporadic disease, FAP and HNPCC. Second, the relationship between BAX mutations, apoptosis and expression of DR4, DR5 and TRAIL was explored in MSI-H tumours.

Materials and methods

Patient and tissue selection

Sporadic patients. Adenomas (n = 74), consecutively removed endoscopically at the Department of Gastroenterology in 1997 of which sufficient material was available to allow serial sectioning for immunohistochemical staining, were selected. MSI-H carcinomas were selected from a previously reported cohort of 500 patients with stage III colon cancer [16]. Sufficient DNA for MSI analysis was available from 273/500 specimens. MSI analysis was performed using the ABI Prism 377 DNA sequencer (Promega, Madison WI, USA) when tumour and matching normal tissue were avaliable. In cases without normal tissue, the HNPCC MSI kit was employed (Roche, Basel, Switzerland), containing 5 previously described consensus markers [17]. MSI-H, defined as instability in ≥ 3/9 or ≥ 2/5 markers respectively was detected in 44/273 samples (16 %). From 27 of these 44 samples, paraffin embedded tissue sections were available to allow serial sectioning for immunohistochemistry. As MSS controls, a series of sporadic carcinomas (n = 29) previously analysed [10] was studied. MSI was excluded in this series by immunohistochemical staining for MLH1 expression [18].

FAP patients. Data from all patients (14 males, 18 females) with classical FAP treated at

the University of Groningen Medical Centre from 1970 to December 2001 were reviewed. All available slides from colectomy specimens and endoscopically removed adenomas were revised. When adenomas showed similar growth patterns and degree of dysplasia in a single patient, one adenoma per patient was studied. Otherwise, additional adenomas were studied. In total, 4 carcinomas and 41 adenomas were included.

HNPCC patients. HNPCC patients had a germline mutation in one of the MMR genes and/or fulfilled the Amsterdam II criteria. All colorectal adenomas and carcinomas removed between 1979 and 2002 with sufficient material available to allow serial sectioning for DNA extraction and immunohistochemistry were selected. In total, 21 carcinomas were examined, 18 from mutation carriers (10 hMSH2, 5 hMLH1, 3 hMSH6) and 3 from patients fulfilling the Amsterdam II criteria. Fifty adenomas, 21 from proven mutation carriers, were analysed. Only HNPCC carcinomas displaying the MSI-H phenotype (n = 15), assessed as described above, were subjected to BAX mutation analysis.

Histopathological classification

Histopathological classifications were performed with hematoxylin-eosin stained slides according to the WHO criteria [19]. For adenomas, circumferential size was measured. Adenomas were classifed as tubular, tubulovillous, villous or serrated. As the group of serrated adenomas was relatively small (n = 4), they were not separately analysed. For statistical purposes, adenomas with tubulovillous or villous histology were combined. Data concerning tumour localisation were retrieved from pathology reports and/or endoscopy reports. Tumours were defined as right-sided (coecum, ascending or transverse colon) or left-sided (descending and sigmoid colon, rectum).

Immunohistochemistry

For immunohistochemical staining, serial 3 µm-thick-sections were cut from paraffin blocks. After deparaffinisation, blocking of endogenous peroxidase with 0.3 % hydrogen peroxide and incubation with avidin and biotin blocking solutions (Vector Laboratories, Burlingame, CA, USA) the primary antibodies were applied for 1 h at room temperature. Staining and control procedures for DR4, DR5 and TRAIL were performed as previously described [10]. Apoptosis was assessed by M30 immunoreactivity, based on the detection of cleaved cytokeratin-18, which is expressed during early apoptosis of epithelial cells [20]. The method has been validated against the gold standard of apoptosis detection by morphological criteria [20]. Staining procedures for M30 were carried out as previously described [20]. MLH1 staining was carried out with a mouse monoclonal antibody (1:100, PharMingen, San Diego, CA, USA).

Staining was evaluated by light microscopy by two investigators, with re-evaluation under a multi-headed microscope if results did not agree. For DR4, DR5 and TRAIL, the percentage of staining cells was estimated semiquantitatively. Samples with staining in more than 10 % of cells were regarded as positive. Apoptosis was assessed in at least 1000 epithelial cells and expressed as a percentage of the total number of cells counted (apoptotic index). Intra and inter-observer variability were less than 10 %.

Table 1. Patient and tumour characteristics.

		Sporadic			FAP		HNPCC	
		Ad *	Ca MSS *	Ca MSI-H *	Ad	Ca	Ad	Ca
N		74	29	27	41	4	50	21
Male (%)		33	38	66	40	50	46	83
Age (yrs, median, range)		65 (28-89)	65 (40-88)	64 (26-76)	30 (11-52)	43 (21-52)	48 (30-67)	54 (31-77)
Size (mm, median/range)		6.0 (3.0-45.0)	-	-	11.0 (2.2-39.6)	-	4.0 (2.0-30.0)	-
Tubular (%)		55	-	-	49	-	68	-
HGD (%)		24	-	-	46	-	44	-
Localisation** (%)	1	28	38	82	7	25	42	57
	2	72	62	18	79 #	75	54 #	43
Tumour stage (%)	I/II	-	38	0	-	75	-	86
	III/IV	-	62	100	-	25	-	14
Differentiation (%)								
	Poor	-	20	52	-	25	-	24
	Good/moderate	-	80	48	-	75	-	76

* Ad = adenoma; Ca = carcinoma; MSS = microsatellite stable; MSI-H: microsatellite instability-high; HGD: high-grade dysplasia; ** localisation: 1 = right; 2 = left; # unknown in number of cases

BAX mutation analysis

From 27 sporadic MSI-H carcinomas and 15 HNPCC-associated MSI-H carcinomas, sufficient DNA could be extracted from microdissected sections of paraffin embedded samples with the Qiaquick PCR purification kit (Qiagen Inc, Chatsworth, CA, USA). PCR was performed using previously published primer sequences [21], amplifying a 94-base pair DNA fragment. PCR was carried out for 32 cycles, each cycle consisting of denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C and extension for 1 min at 72 °C. PCR products were visualised on a 1.5 % agarose gel and subsequently subjected to direct sequencing using the ABI Prism™ genetic analyser (Applied Biosystems Product, Foster City CA, USA).

Statistical analysis

Appropriate tests were used to assess differences in patient and tumour characteristics (chi-square test) and immunohistochemical findings (Mann-Whitney test for continuous variables, chi-square test for discontinuous variables). Correlations between percentages of positive staining and apoptotic indices were calculated with the Spearman test. P-values < 0.05 were considered significant. SPSS for Windows software was used in all statistical analyses.

Table 2. DR4, DR5 and TRAIL expression patterns in sporadic, FAP and HNPCC adenomas.

	Sporadic	FAP	HNPCC
DR4 pos (n) #	69/74 (93 %)	38/41 (93 %)	49/50 (98 %)
% DR4 pos cells *	35 (15-100)	65 (15-100)	50 (20-100)
DR5 pos (n) #	74/74 (100 %)	40/41 (98 %)	48/50 (96 %)
% DR5 pos cells *	90 (20-100)	90 (40-100)	100 (20-100)
TRAIL pos (n) #	58/74 (78 %)	32/41 (78 %)	35/50 (70 %)

number of cases with positive staining relative to the number of samples investigated
* median (range) percentage of positively staining cells among positive samples

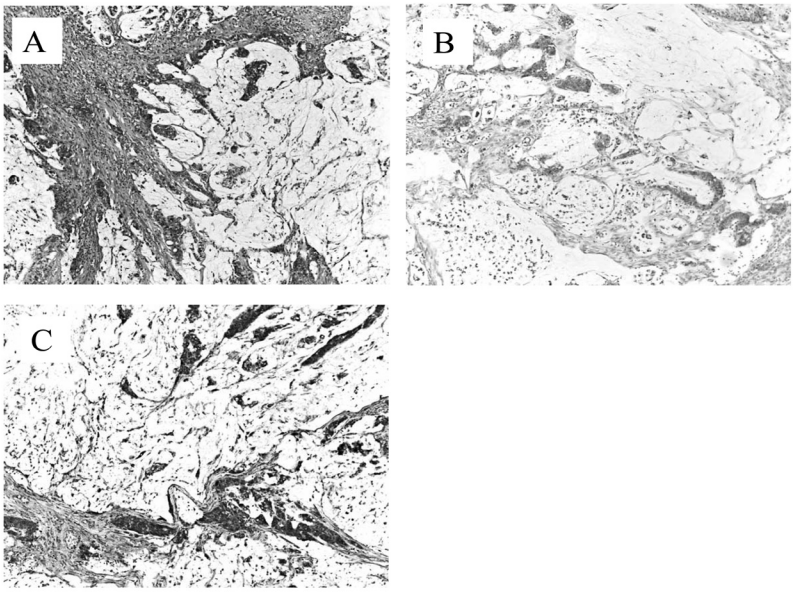

Figure 1. DR4 and DR5 expression in mucinous adenocarcinoma. Hematoxylin-eosin (A), DR4 (B) and DR5 staining (C) showing absence of DR4 expression with intact DR5 expression. See appendix for colour pictures.

Table 3**.** DR4, DR5 and TRAIL expression patterns in sporadic, FAP and HNPCC carcinomas.

	Sporadic MSS	Sporadic MSI-H	FAP	HNPCC
DR4 pos (n) #	29/29 (100 %)	21/27 (78 %)	4/4 (100 %)	21/21 (100 %)
% DR4 pos cells *	100 (50-100)	95 (20-100)	90 (75-100)	100 (20-100)
DR5 pos (n) #	29/29 (100 %)	27/27 (100 %)	4/4 (100 %)	21/21 (100 %)
% DR5 pos cells *	100 (60-100)	100 (20-100)	100 (95-100)	100 (20-100)
TRAIL pos (n) #	21/29 (72 %)	10/27 (37 %)	3/4 (75 %)	17/21 (81 %)

number of cases with positive staining relative to the number of samples investigated

* median (range) percentage of positively staining cells among positive samples

Results

Patient and tumour characteristics

Patient and tumour characteristics are summarised in table 1. Inherent to the patient groups, several differences were observed between groups. Median age of patients with sporadic tumours was higher than FAP and HNPCC cases ($p < 0.001$). Median adenoma size was smaller in HNPCC compared to FAP and sporadic cases ($p < 0.001$). HNPCC-associated carcinomas and MSI-H sporadic carcinomas were more often localised in the proximal colon compared to FAP and sporadic MSS cases. Tumour stage was lower in HNPCC and FAP-associated cases than in their sporadic MSS counterparts ($p < 0.001$).

Expression of DR4, DR5 and TRAIL and apoptosis in adenomas

Immunohistochemical staining results are summarised in table 2. DR4 and DR5 expression was positive in virtually all adenomas. Among positive adenoma samples, median percentages of positively staining cells were similar in patient groups and independent of histopathological characteristics such as size, growth type or degree of dysplasia. Staining patterns of DR4, DR5 and TRAIL expression were generally heterogenous throughout adenoma tissue, with no consistent co-localisation for the different proteins. Adenomas with absence of DR4 or DR5 expression were not characterised by distinct histopathological features. There were no adenomas staining negative for both DR4 and DR5. TRAIL expression was positive in about 75 % of adenomas, independent of the patient group, and not associated with any histopathological parameter. Strikingly, 8/9 adenomas with absent DR4 expression also stained negative for TRAIL.

Apoptotic indices, assessed by M30 immunoreactivity, were similar in patient groups. There was a positive correlation between apoptotic index and DR4 positivity ($r = 0.23$, $p < 0.01$) and TRAIL positivity ($r = 0.18$, $p = 0.02$). This correlation was not observed for DR5.

Table 4. Apoptosis, DR4, DR5 and TRAIL expression in MSI-H carcinomas with wild-type or mutant BAX.

	BAX mutant (n=6)	Wild-type BAX (n=36)	p
Apoptotic index + (%)	1.4 ± 0.9	1.2 ± 0.2	ns
DR4 pos (n) #	4/6	32/36	ns
% DR4 pos cells *	100 (50-100)	100 (20-100)	ns
DR5 pos (n) #	6/6	36/36	ns
% DR5 pos cells *	95 (60-100)	100 (20-100)	ns
TRAIL pos (n) #	3/6	19/36	ns

+ expressed as mean ± SEM.# number of cases with positive staining relative to the number of samples investigated.* median (range) percentage of positively staining cells among positive samples. ns: not significant

Expression of DR4, DR5 and TRAIL and apoptosis in carcinomas

Results are summarised in table 3. DR4 expression was positive in almost all carcinomas, with similar percentages of positive cells among positive samples in the different patient groups. Staining was generally homogenous throughout tumour tissue. Six out of 27 sporadic MSI-H carcinomas, all with mucinous histology, were DR4 negative (figure 1). Four other mucinous carcinomas, also MSI-H, stained positive for DR4. DR5 expression was positive in all carcinomas, including those negative for DR4 (figure 1). Percentages of positively staining cells among positive samples in the various groups were similar. Staining results for DR4 or DR5 were independent of tumour stage, localisation or degree of differentiation.

TRAIL expression was positive in 37-81 % of the carcinomas, depending on the patient group. Similar to the observation in adenomas, 5/6 carcinomas with absence of DR4 expression also stained negative for TRAIL. As in adenomas, there was no apparent co-localization of DR4, DR5 and TRAIL expression. There was a positive correlation between apoptotic index and DR4 positivity ($r = 0.39$, $p < 0.001$) and DR5 positivity ($r = 0.18$, $p = 0.01$), but not with TRAIL positivity.

BAX mutation analysis and the relationship with apoptosis, DR4, DR5 and TRAIL expression

Frameshift mutations in the G(8) repeat of the BAX gene were detected in 2/15 (13%) MSI-H HNPCC-associated carcinomas and 4/27 (15 %) MSI-H sporadic carcinomas. Sequence analyses of BAX mutations showed a 1 bp deletion in 5 cases and in 1 case an insertion of 1 bp.

Apoptosis and expression patterns of DR4, DR5 and TRAIL were compared between MSI-H tumours with (n=6) and without (n=36) BAX gene mutations, depicted in table 4. Apoptotic indices were comparable between both groups. DR4, DR5 and TRAIL was observed with similar frequencies in BAX mutant and wild-type BAX tumours. Median percentages of DR4 and DR5 positivity also did not differ between these groups.

Discussion

The present study shows extensive expression of the TRAIL death receptors DR4 and DR5 in sporadic and hereditary colorectal neoplasms. Moreover, no support was found for a critical role for BAX gene mutations as an apoptosis-evading mechanism in MSI-H tumours. Expression patterns of DR4 and DR5 in sporadic neoplasms were in accordance with previous findings [10,11]. A novel finding was that a subset of sporadic carcinomas, all MSI-H and with mucinous histology, showed no DR4 expression. The background for this finding is unclear. The gene for DR4 is located on chromosome 8p21, a region associated with frequent loss of heterozygosity (LOH) in CRC development [22,23]. However, 8p LOH is not particularly related with mucinous histology or MSI [22,23]. Mutations in the DR4 gene have been described in several tumour types [8,9] but have not yet been studied in CRC. Alternatively, it may be that absence of DR4 expression in MSI-H tumours occurs as a result of hypermethylation.

Interestingly, in the present study, absent DR4 expression was associated with absent TRAIL expression in the majority of cases. On the other hand, absent TRAIL expression was more often encountered in our study than absent DR4 expression, a finding also reported by others [11]. Future studies may be directed at determining the mechanisms behind absence of TRAIL and DR4 expression in certain colorectal tumours. It must be realised however that the technique of immunohistochemistry may not be sensitive enough to determine TRAIL and DR4 expression. The importance of absent or low levels of endogenous TRAIL expression in colon epithelial cells for induction of apoptosis is unknown. Other cells such as macrophages and NK cells are known to express TRAIL, which may play a role in TRAIL-induced apoptosis. For example, TRAIL-expressing macrophages isolated from pleural effusions of cancer patients induced apoptosis in colon cancer cells [24]. It is therefore important to stress that DR4 and DR5 expression was observed in almost every colorectal neoplasm, with no tumour being negative for both receptors, suggesting that neoplastic cells are prone to TRAIL induced apoptosis. The fulfilment of this prerequisite offers hope for the future use of rhTRAIL, agonistic TRAIL-receptor antibodies or other, yet to be developed, TRAIL agonists. Data on preclinical activity profiling of these agents are promising [25]. TRAIL induces apoptosis in a wide variety of cancer cell lines of diverse origins, including colon cancer, while having little or no detectable cytotoxic effect on normal cells in vitro and in vivo [8]. In cynomolgus monkeys and chimpanzees, repeated intravenous injections of native rhTRAIL did not cause detectable toxicity [26,27]. The combination of chemotherapy and rhTRAIL potentiated antitumour activity in human colon cancer cell lines as well as in xenografted mice [26,28,29]. In addition, specific targeting of DR4 and DR5 with monoclonal agonistic antibodies exerted similar effects as TRAIL in vitro and in vivo [30,31]. Phase I trials using rhTRAIL as well as monoclonal agonistic antibodies are underway. Targeting of death receptors may even be useful at a premalignant stage given our finding of pro-apoptotic death receptor expression in adenomas, for example in chemopreventive regimens for FAP or HNPCC patients. Several studies indicate that TRAIL act synergistically with NSAIDs in induction of apoptosis [15,32,33], which may be a potentially useful combination regimen, deserving further study.

Recent studies in cell lines suggested that the presence of functional BAX is important

in determining the outcome of TRAIL-based therapeutic regimens [12-15]. BAX is frequently mutated in mismatch repair deficient tumours [2]. It was shown that BAX-deficient human colon carcinoma cells were resistant to death-receptor ligands, whereas BAX-expressing sister clones were sensitive [12-15]. In search for evidence for such a mechanism, we investigated whether apoptotic indices, DR4, DR5 and TRAIL expression were related to the presence or absence of BAX mutations in MMR-deficient, MSI-H tumours. We found similar apoptotic indices in BAX mutant and wild-type cases. Although the number of tumours investigated was quite small, the results are in accordance with recent data from others [34]. The small number of BAX mutations in our study does not allow definite conclusions on the role of BAX. Nevertheless, our data suggest that BAX mutations in these tumours do not protect completely against apoptosis. One explanation may lie in the following. TRAIL can induce apoptosis by direct activation of caspases (extrinsic, type I pathway), but also by activation of the mitochondrial (intrinsic, type II) pathway with subsequent release of factors such as cytochrome c [15]. It seems that in some cell types, both pathways are activated, while in others, only one pathway is preferentially activated [9]. One could speculate that the mitochondrial pathway, which involves BAX, is less important than the extrinsic pathway in colorectal cancer cells. Alternatively, it may be that other ligands than TRAIL, e.g. FasLigand, may be more important mechanisms in apoptosis induction in these tumours. The finding of almost ubiquitous expression of DR4 and DR5 in MSI-H tumours together with a low prevalence of BAX mutations, does not suggest a survival benefit for BAX mutated cells in the setting of MMR deficiency. Taken together, we found no evidence supporting the concept of BAX inactivation as a critical mechanism to evade TRAIL-receptor-mediated apoptosis in vivo.

Whether TRAIL-mediated apoptosis plays a role in colon cancer cells in vivo remains to be proven. There are however some observations supporting the functionality of this pathway. First, studies using TRAIL-deficient mice demonstrated that TRAIL is important in controlling tumour growth and metastasis [35,36]. Second, sensitivity to TRAIL-induced apoptosis in colon cancer cell lines correlated with the relative expression of DR4 and DR5 on the cell membrane [37]. Third, TRAIL expressing macrophages isolated from pleural effusions of cancer patients induced apoptosis in colon cancer cells in a concentration dependent fashion [24]. Finally, in our study, apoptotic indices were positively correlated to DR4 positivity, both in adenomas and carcinomas, and to DR5 positivity in carcinomas.

In conclusion, the widespread expression of pro-apoptotic death receptors for TRAIL in sporadic and hereditary colorectal neoplasms provides potential targets for apoptosis induction. In addition, we found no support for BAX inactivation as a mechanism to evade apoptosis in vivo in MSI-H tumours. These observations hold promise for targeting of DR4 or DR5 with TRAIL, agonistic antibodies or other TRAIL-receptor agonists to treat or prevent colorectal neoplasms.

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