Contribution of Epigenetic Silencing of Tumor Necrosis Factor–Related Apoptosis Inducing Ligand Receptor 1 (DR4) to TRAIL Resistance and Ovarian Cancer

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Abstract

Dysregulation of apoptosis may support tumorigenesis by allowing cells to live beyond their normally intended life span. The various receptors for tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) are located on chromosome *8p21.2*, a region frequently deleted in ovarian cancer. Lack of expression of TRAIL receptor 1 (death receptor 4, DR4) correlates with resistance to TRAIL-induced apoptosis in ovarian cancer cells. Reconstitution of DR4 in the TRAIL-resistant A2780 ovarian cancer cell line was investigated with the demethylating agent 5-aza-2′-deoxycytidine and transient gene transfer. Regulation of other genes in the TRAIL pathway by 5-aza-2′-deoxycytidine was assessed in DNA GeneChip experiments. Primary ovarian cancers were analyzed by methylation-specific PCR and immunohistochemical analysis of a tissue microarray. Regulation of DR4 expression by demethylation or transient transfection is of functional relevance for TRAIL resistance in an ovarian cancer cell line. Hypermethylation of the *DR4* promoter could be found in 10 of 36 (27.7%) DNAs isolated from ovarian cancer tissue. In an independent set of 68 ovarian cancer cases, a complete loss or down-regulation of DR4 protein expression was observed 10.3% and 8.8% patients, respectively. A significant ($P = 0.019$) majority of these patients was below 50 years of age. Our findings show a functional relevance of the level of DR4 expression in ovarian cancer and suggest a substantial contribution of *DR4* hypermethylation and consequent loss of DR4 expression to ovarian cancer pathogenesis, particularly in premenopausal patients. (Mol Cancer Res 2005; 3(6):335–43)

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Introduction

Defects in programmed cell death (apoptosis) mechanisms play an important role in tumor pathogenesis by enabling neoplastic cells to escape from cell autonomous or immunologic growth control mechanisms (1). Players in the various apoptotic pathways are well-known oncogenes but also intriguing candidates for tumor suppressor genes. A considerable amount of effort has been put into the mutational analysis of receptors for apoptosis-inducing proteins, including those for tumor necrosis factor (TNF)–related apotosis inducing ligand (TRAIL), but definite mutations are rare. The functional TRAIL receptors death receptors 4 (*DR4, TRAIL-R1*) and 5 (*DR5, TRAIL-R2/TRICK2/KILLER*), and decoy receptors 1 (*DcR1, TRAIL-R3/TRID*) and 2 (*DcR2, TRAIL-R4/*TRUNDD) with thus far little known physiologic function are all located on chromosome *8p21.2* according to the University of California Santa Cruz Human Genome Project Working Draft June 2002 (http://genome.cse.ucsc.edu). This finding suggests evolutionary multiplication of one gene in this region and subsequent gain or loss of function as mediator or inhibitor of apoptosis, respectively. Besides other common areas of genetic variation in ovarian cancer (reviewed in ref. 2), *8p21.2* is a region frequently deleted in this malignancy (3, 4). More recently, it was observed that ovarian cancers have a higher TRAIL expression than normal ovarian epithelial samples and that high expression of TRAIL is associated with prolonged survival (5), additionally implicating a potential role of the TRAIL system in the pathogenesis of ovarian cancer. However, definite mutations in TRAIL receptors in ovarian cancer could not be detected (6). Therefore, we investigated a number of ovarian cancer cell lines for expression of players in the TRAIL pathway and could show that the lack of expression of DR4 correlated with resistance to TRAIL, whereas the expression of other receptors did not (7). This phenomenon has also been described by other groups in different tumor entities as part of their efforts to characterize TRAIL as a potential therapeutic agent (8, 9). We wanted to further explore our observation for its true physiologic relevance on a more mechanistic level, and test whether the lack of expression of DR4 in ovarian cancer cell lines is a genetic or epigenetic phenomenon, because a variety of tumor suppressor genes have been shown to be silenced in human cancers (10). Epigenetic silencing of TRAIL receptors is common in some cancer entities (11, 12), although hypermethylation of *DR4* and *DR5*

in ovarian cancer has not been studied to date. Hypermethylation of the promoters of these genes could be causally involved in tumorigenesis of ovarian cancer. A reversion of DR4 loss in ovarian cancer by demethylation, together with the observation that methylation of *DR4* can be found in a significant number of clinical cases, would provide an important piece of the puzzle in the poorly understood field of ovarian cancer biology. This observations would also guide the use of TRAIL as one of the emerging tools for future apoptosis-targeted cancer therapies.

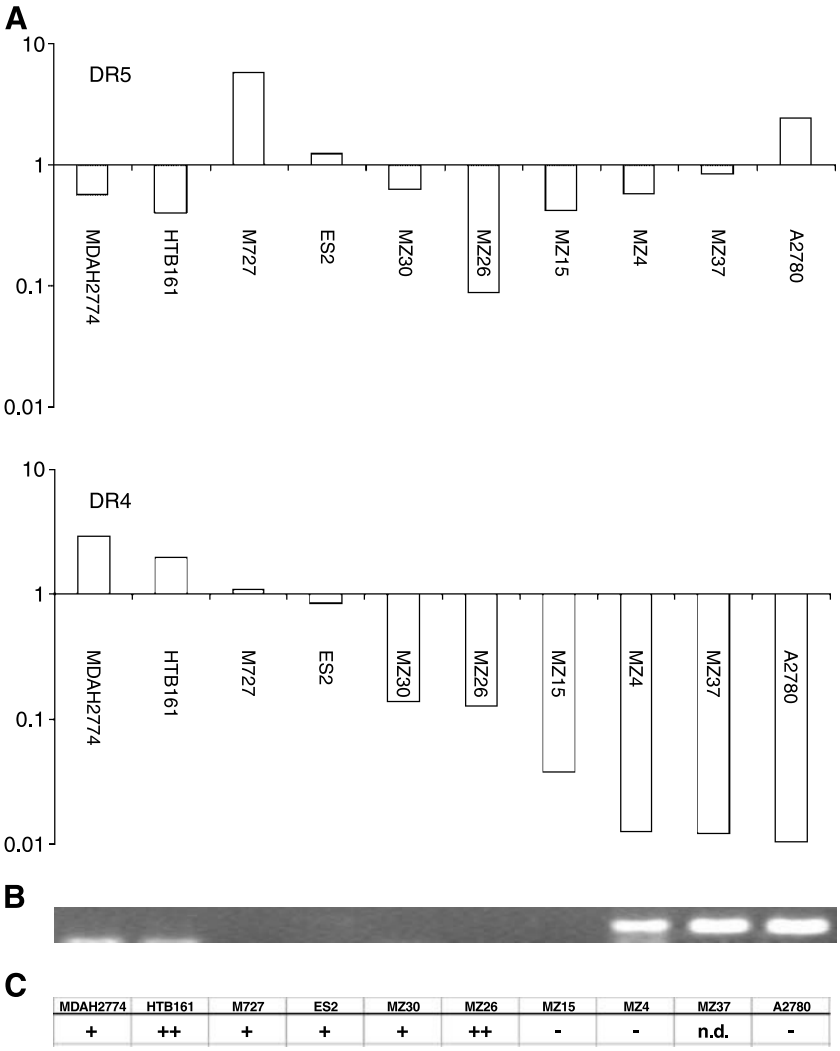
Results

DR4 and DR5 Expression in Ovarian Cancer Cell Lines

We examined a panel of ovarian cancer cell lines and determined the *DR4* and *DR5* mRNA expression using real-time PCR. We found that two cell lines (A2780 and MZ-37) had no detectable *DR4* expression and two other cell lines had a very low *DR4* expression (MZ-4 and MZ-15; Fig. 1A). Then, the methylation status was determined and correlated with the expression pattern. We observed *DR4* promoter hypermethylation in 3 of 4 (75%) cell lines displaying low or missing *DR4* expression (Fig. 1B). In contrast, *DR4* hypermethylation could not be found in any of the remaining *DR4*-expressing cell lines. *DR5* expression was rather uniformly present throughout the cell lines and hypermethylation of the *DR5* promoter was not observed (data not shown).

Ovarian Cancer Cells Are Resistant to TRAIL

We tested the nine available cell lines for TRAIL sensitivity. The cell lines with low DR4 expression levels showed to be more resistant to the apoptotic effects of TRAIL at concentrations of 1,000 ng/mL (Fig. 1C). Ovarian cancer cell line A2780 was completely resistant to TRAIL-induced apoptosis and previous experiments prompted us to assume that TRAIL resistance is caused by lack of DR4 expression (6). Incubation of A2780 cells with 5-aza-2′-deoxycytidine (AzadC) restored DR4 expression and sensitized A2780 cells to subsequent treatment with TRAIL (Fig. 2A). In accordance with these two facts, AzadC induces time-dependent *DR4* mRNA reexpression in the A2780 cell line, reaching a peak of expression comparable with the constitutively DR4-expressing, TRAIL-sensitive cell lines after 96 hours (Fig. 2B).



MDAH2774	HTB161	M727	ES2	MZ30	MZ26	MZ15	MZ4	MZ37	A2780
+	++	+	+	+	++	-	-	n.d.	-

FIGURE 1. AzadC treatment demethylates hypermethylated CpG islands of the *DR4* promoter and induces *DR4* reexpression, thus sensitizing A2780 cells to TRAIL-induced apoptosis. **A.** Apoptosis assay. Combined treatment with AzadC and TRAIL leads to an increase in apoptosis as measured with APO-Direct kit. **B.** Time-dependent up-regulation of *DR4* mRNA by AzadC (relative quantitative reverse transcription-PCR) in the ovarian cancer cell line A2780. **C.** Apoptosis after TRAIL treatment (100 ng/mL) in A2780 ovarian cancer cells transfected with either truncated or full-length *DR4* compared with mock-treated cells.

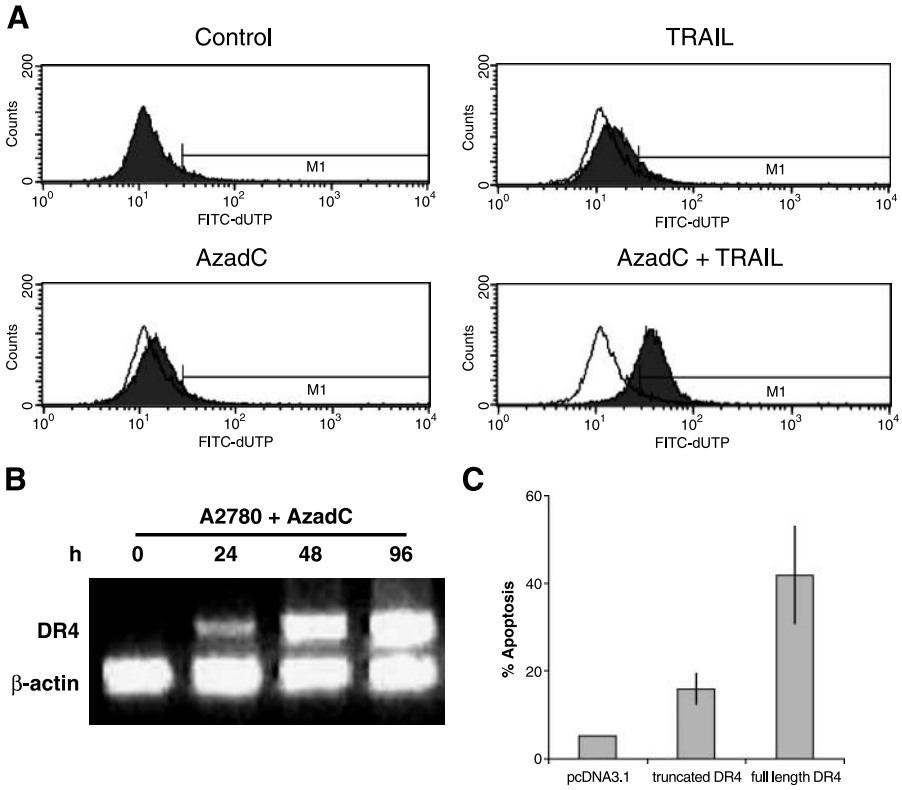


FIGURE 2. **A.** Real-time DR4 and DR5 expression related to an internal calibrator (set to 1). PCR reactions of the A2780 and MZ-37 cell lines yielded no detectable product after 40 PCR cycles. **B.** Methylation status of the *DR4* promoter in ovarian cancer cell lines. **C.** TRAIL sensitivity of ovarian cancer cell lines. TRAIL (1,000 ng/mL)–induced apoptosis was assessed as follows: negative (–) for <10% positively stained cells; positive (+) for 10% to 50% apoptosis; and strongly positive (++) for >50% apoptotic cells after 24-hour incubation.

Microarray Analysis of AzadC-Treated Cells

Regulation of other players in the TRAIL pathway besides DR4 can also be observed and may, in addition, be responsible for the reconstitution of TRAIL sensitivity in the A2780 cell line. To investigate these genes, we did a global gene expression analysis comparing mock- and AzadC-treated A2780 cells by standard microarray technology. We focused on the expression of genes involved in the known TRAIL pathways and checked all players for their representation on the Affymetrix HG-U133A and HG-U133B GeneChip (Table 1). All genes of interest were well represented except, interestingly, *DR4*, where only 2 of 11 probes from the probe set on GeneChip HG-U133B (231775_at derived from an EST) are complementary to the *DR4* mRNA (NM_003844); therefore, values for this probe set were not used in Table 1. No significant regulation could be detected in any of the analyzed genes, making us even more confident that the observed induction of *DR4* by demethylation is responsible for the reversed TRAIL sensitivity (Table 1).

Transfer of the DR4 *Gene into the A2780 Cell Line*

To further confirm the pivotal role of DR4 in TRAIL-induced apoptosis, we transiently transfected A2780 cells with a full-length *DR4* construct and a truncated variant, which lacks the functionally essential death domain and compared them to mock-treated cells. Apoptosis detection was done using an annexin assay, which enabled us to evaluate early apoptosis and to obtain a set of appropriate data in a transient expression setting. A2780 cells were sensitized to TRAIL by transient reconstitution of a full-length *DR4* construct comparable with demethylation by AzaD (Fig. 2C). Interestingly, also, the overexpression of the truncated variant of the DR4 protein—missing the highly conserved death domain but retaining the possibility to form heterodimers with other receptors—exhibited some residual proapoptotic effects.

DR4 *Methylation and Expression in Primary Cancer Samples*

Tumor DNA of 36 ovarian cancer cases were investigated and 10 of 36 (27.8%) patients showed methylation of *DR4* in their tumor (Fig. 3). We extended our study to a population of 68 ovarian cancer patients (Table 2) for whom clinical information and archival paraffin material was available. Not unexpectedly, DR4 was detectable in the ovarian stromal tissue of virtually all patients by immunohistochemistry (Fig. 4), because contrary to TNF-α and FasL, TRAIL and its receptors are constitutively expressed in many tissues, lacking any apoptotic effect under normal conditions. Seven of 68 (10.3%) patients showed complete loss of DR4 expression in epithelial tumor tissue, in accordance with the data from the methylation studies (Fig. 4C). An additional six patients (8.8%) showed marked reduction in DR4 expression as confirmed independently by two individual pathologists (Fig. 4B).

Loss or Down-Regulation of DR4 Expression Correlates with Age

Interestingly, loss or down-regulation of DR4 expression in ovarian cancer correlated significantly ($P = 0.019$, Pearson's χ^2)

Table 1. Whole-Genome Gene Expression Study Using Affymetrix GeneChips HG-U133A and HG-U133B

Probe Set	Gene Title (Gene Symbol)	Chromosomal Location	Relative Expression	
			Before AzadC	After AzadC
202687_s_at	TNF-related apoptosis-inducing ligand (*TRAIL*)	3q26	AA	AA
202688_at			1.0	2.1
231775_at*	Death receptor 4 (*DR4*)	8p21	N.a.*	N.a.*
209294_x_at	Death receptor 5 (*DR5*)	8p22-p21	1.0	1.1
209295_at			1.0	1.4
210405_x_at			1.0	1.4
210484_s_at	Decoy receptor 1 (*DcR1*)	8p22-p21	AA	PA
206222_at			AA	PA
211163_s_at			AA	AA
210654_at	Decoy receptor 2 (*DcR2*)	8p21	PA	PA
204932_at	Osteoprotegerin (*OPG*)	8q24	AA	AA
204933_s_at			AA	AA
207686_s_at	Caspase 8 (*CASP8*)	2q33-q34	AA	AA
213373_s_at			1.0	1.4
202535_at	Fas-associated via death domain (*FADD*)	11q13.3	1.0	1.8
208485_x_at	CASP8 and FADD-like apoptosis regulator (*CFLAR*, *FLIP*)	2q33-q34	1.0	1.0
211316_x_at			1.0	1.3
211317_s_at			AA	PA
211862_x_at			1.0	1.0
1729_at	TNFRSF1A-associated via death domain (*TRADD*)	16q22	1.0	2.1
205641_s_at			AA	AA
204493_at	BH3 interacting domain death agonist (*BID*)	22q11.1	1.0	0.7
211725_s_at			1.0	1.0
204413_at	TNF receptor-associated factor 2 (*TRAF2*)	9q34	MA	AA
203684_s_at	B-cell CLL/lymphoma 2 (*BCL2*)	18q21.3	AA	AA
203685_at			PP	MA
207004_at			PM	AA
207005_s_at			AA	AA
202763_at	Caspase 3 (*CASP3*)	4q34	1.0	1.0
209790_s_at	Caspase 6 (*CASP6*)	4q25	1.0	0.9
207181_s_at	Caspase 7 (*CASP7*)	10q25	1.0	1.2

NOTE: Data represent genes involved in the TRAIL-regulated apoptotic pathway with relative expression data (fold differences) before (set to 1.0) and after AzadC treatment. Relative expression is absent (A), marginal (M), and present (P) by the Affymetrix detection algorithm. Results represent means of duplicate experiments.
*Probe set not appropriate for *DR4*.

with the age of the patients: younger patients, who developed their ovarian cancer in their reproductive years (below 50), had a much higher probability of loss or down-regulation of DR4 expression compared with postmenopausal women, 8 of 21 (38.1%) versus 5 of 47 (10.6%).

Discussion

The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell attrition. Programmed cell death—apoptosis—represents a major source of this attrition. The evidence is mounting, principally from studies in mouse models and cultured cells as well as from descriptive analyses of biopsied stages in human carcinogenesis, that acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer (1).

Of the five TRAIL receptors identified in humans, four belong to the TNF receptor family; all four of them, *DR4* (*TRAILR1*), *DR5* (*TRAILR2*), *DcR1* (*TRAILR3*), and *DcR2* (*TRAILR4*), are located on *8p21* in close vicinity. *8p21* is a genomic region with frequent allelic loss in ovarian cancer (3, 4). DR4 and DR5 contain a stretch of 95 amino acids in the cytoplasmic region designated as the death domain. This highly conserved functional domain can trigger an apoptotic response upon binding of the receptor to its ligand TRAIL. DcR1 lacks a death domain and is, therefore, unable to transmit an apoptotic signal when binding with TRAIL occurs (13). Consequently, the overexpression of DcR1 reduces the sensitivity to TRAIL-induced apoptosis, which might be a physiologic regulatory function of this protein (14, 15). Less clear is the case for DcR2, which has a truncated death domain retaining about one third of the death domain motif and is unable to induce apoptosis via the activation of the NF-κB–mediated survival pathway (16, 17), but other studies have reported the opposite result (18). However, in response to the activation of cell death receptors with an intact death domain, DR4 and DR5, the extrinsic pathway of apoptosis is triggered and results in the formation of a death-inducing signaling complex.

Are DR4 and DR5 functionally redundant? We observed in a previous investigation that the soluble human recombinant TRAIL protein can efficiently trigger apoptosis only in DR4-positive cells, regardless of the remaining receptor status, especially of the apoptosis-inducing receptor DR5 (7). In accordance with our data, deletions on chromosome *8p* within the coding region of the DR4 receptor were found to abolish the effects of TRAIL even in the presence of DR5 (19) and missing expression of DR4 correlated significantly with TRAIL resistance in various tissues (8, 9), whereas the expression of other TRAIL receptors did not. Abdollahi et al. (20) showed that interleukin-8 turned a TRAIL-sensitive ovarian cancer cell line (OVCAR3) into a TRAIL-resistant one, mainly by decreasing DR4 expression with unchanged expression of the other TRAIL-binding receptors (DR5, DcR1, and DcR2).

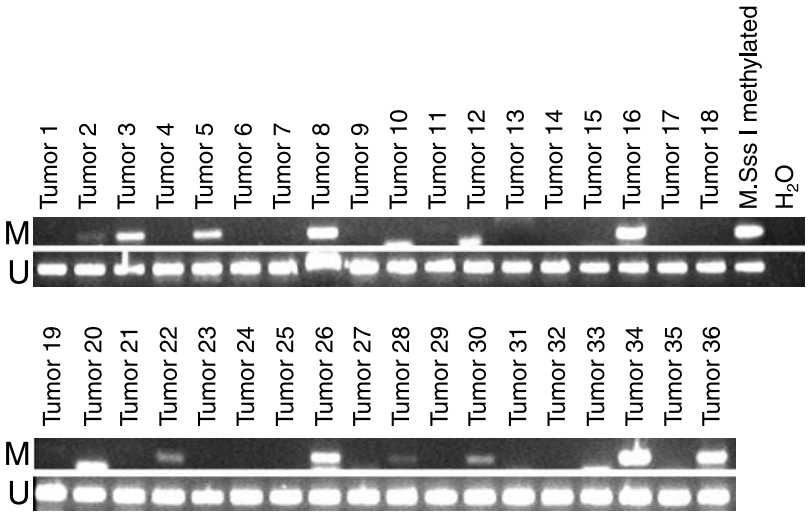


FIGURE 3. Methylation analysis of the *DR4* gene of total DNA from 36 ovarian cancer tissues using methylation-specific PCR. As positive control, *in vitro* M.Sss I methylated human DNA was used. M, PCR products with primers recognizing methylated sequences; U, PCR products with primers recognizing unmethylated sequences.

A reduction of DR4 expression in ovarian cancer may be caused by epigenetic silencing or allelic loss. Epigenetic and genetic events may aid tumorigenesis according to Knudson's two-hit hypothesis, sometimes in a concerted action by sequential elimination of gene function in both alleles (10). We could show that epigenetic down-regulation of DR4 in an ovarian cancer cell line leads to TRAIL resistance and its reconstitution by demethylation restores sensitivity to human soluble recombinant TRAIL, without an influence on the expression of any other known player of the apoptotic TRAIL pathway by the demethylation process. Consequently, reconstitution by transient transfection of *DR4* also restored sensitivity of this cancer cell line to TRAIL (Fig. 2C). Interestingly, overexpression of a truncated DR4 receptor without a death domain also led to a slightly increased sensitivity to TRAIL most probably due to the fact that the truncated receptor is still able to bind to its ligand, resulting in cross-linking with available wild-type receptors in the cell. Our functional data support the importance of the regulation of DR4 expression levels and make a functional redundance between DR4 and DR5 hard to argue.

Are these *in vitro* observations relevant for ovarian cancer pathogenesis? Functional knockouts of tumor suppressor genes by epigenetic silencing or by inactivating mutations are generally considered to accumulate in cultured cancer cell lines over the course of time. Therefore, we decided to extend our investigations from cell lines to primary tumors and consequently studied *DR4* methylation by MSP in primary ovarian cancer cases. *DR4* was methylated in over 20% of primary ovarian cancer cases, which is strong evidence that epigenetic silencing of DR4 in ovarian carcinoma is not a cell culture phenomenon. In further immunohistochemical experiments, we could confirm these data by showing that a corresponding number of ovarian cancer patients have a loss of DR4 protein expression in their tumor. There are also other data emerging that suggest a role for the TRAIL pathway in ovarian cancer. Very recently, it was observed that high expression of TRAIL is associated with prolonged survival in advanced ovarian cancers (5). It unclear, to date, whether the differences in TRAIL expression observed between patients with good and with bad prognosis were due to an up-regulation or down-regulation in tumor cells or in cells of the immune system invading the tumor tissue.

How do the observations of a dysregulated apoptotic pathway fit into current models of ovarian cancer pathogenesis? Between menarche and menopause, in every normal menstrual cycle after the ovum is released, the epithelial damage is closed by proliferation of the neighboring ovarian epithelium, hereby restoring the surface integrity. However, sometimes, ovarian surface epithelium invaginates and forms inclusion cysts in the ovarian stroma, which are considered to be premalignant lesions (21). The frequency of this physiologic progress over an individual woman's lifetime seems to be implicated in ovarian cancer. Epidemiologically, the use of antiovulatory drugs, late menarche, early menopause, breast feeding, and multiple pregnancies, all reducing the number of menstrual cycles, also lower the risk of ovarian cancer. The epithelial cells in these inclusion cysts are exposed to high levels of estrogen that may facilitate ovarian carcinogenesis (22). Interestingly, there is now accumulating evidence that apoptosis may play a part in physiologic elimination of these inclusion cysts (reviewed in ref. 23). TRAIL-induced apoptosis may be engaged in this process as TRAIL is involved in immunosurveillance and scavenging of tumor cells. Our experimental and clinical data support this hypothesis by showing that the expression of DR4, a receptor for a prominent apoptosis-inducing protein, is lost or reduced in a significant number of ovarian cancer patients.

To summarize, disturbances of the TRAIL pathway seem to play an essential role in ovarian cancer pathogenesis. The investigation of the emerging functional significance of TRAIL receptor down-regulation will hopefully increase our understanding of the ovarian cancer pathogenesis and highlights the importance of a detailed investigation of the TRAIL receptor pathway before initiating TRAIL therapy in future.

Table 2.Clinicopathologic Characteristics and Survival

Histology	
Adenocarcinoma	65
Cystadenocarcinoma	57
Serous	42
Endometroid	12
Mucinous	3
Undifferentiated/unclassified	8
Clear cell carcinoma	3
Stage I	15
Ia	6
Ic	10
Stage II	8
IIa	5
IIb	1
IIc	2
Stage III	37
IIIa	3
IIIb	9
IIIc	25
Stage IV	
Age	
<50 y (Ø = 43.2, SD 5.9)	21
≥50 y (Ø = 62.2, SD 8.7)	47
Survival	
≤5 y	20
>5 y	48

Materials and Methods

Ovarian Cancer Cell Lines

A2780 cells were obtained from European Collection of Cell Cultures (Salisbury, Wiltshire, United Kingdom). The ES2, HTB-161, and MDAH-2774 cell lines were purchased from American Type Culture Collection (Rockville, MD). MZ-4, MZ-15, MZ-26, MZ-27, MZ-30, and MZ-37 ovarian cancer cell lines were a kind gift from Robert Zeillinger. All cell lines were cultured in RPMI 1640 with Glutamax (Invitrogen, Carlsbad, CA) supplemented with 10% FCS and penicillin/streptomycin. The cells were kept under standard conditions at 37°C in a humidified 5% CO_2 incubator.

Treatment with TRAIL and/or AzadC

Recombinant human TRAIL was purchased from Chemicon International, Inc. (Temecula, CA). The demethylating agent AzadC was from Sigma-Aldrich Corporation (St. Louis, MO). The cells were plated at low density and allowed to adhere to the plates overnight. The next day, the cells were treated either with freshly prepared AzadC at a final concentration of 10 μmol/L or mock-treated with PBS for ~48 hours and, thereafter, treated with recombinant human TRAIL (100 ng/mL) for 24 hours. For assessing TRAIL sensitivity, concentration of 1,000 ng/mL and incubation time of 24 hours were used, respectively. Cells were assessed for apoptosis after 24 hours and compared with untreated controls.

Reconstitution of Wild-type DR4 in Ovarian Cancer Cell Line A2780

Two PCR products were amplified using the Expand High Fidelity PCR System (Roche, Basel, Switzerland), one of them encompassing the whole coding sequence of the *DR4* gene and a truncated version, omitting the COOH-terminal death domain (amino acids 357-468). Subsequently, PCR products were cloned into the pcDNA3.1+ vector using restriction enzymes sites provided in oligonucleotides. The absence of mutations was confirmed by sequencing. Transient transfection was carried out using the SuperFect reagent (Invitrogen), incubating the A2780 cells with DNA/SuperFect reagent ratio of 1:10 for 2 hours. Twenty-four hours after transfection, the cells were subjected to further incubation with TRAIL (100 ng/mL) for another 24 hours. All experiments were done in duplicate and results are given by the percentage of apoptotic cells. The percentage was calculated by comparing the relation of transfected, ligand-treated cells versus transfected, untreated controls. Apoptosis induction was measured by Annexin V-FITC apoptosis detection kit (Alexis, Lausen, Switzerland), which detects phosphatidylserine on the outer surface of the cell membrane and assesses early stages of apoptosis. Flow cytometry was used to measure the percentage of positively stained cells.

DNA Fragmentation Assay Analyzed by Flow Cytometry

Using the Apo-Direct kit (Phoenix Flow Systems, San Diego, CA) the 3′ OH termini in DNA breaks were measured by attaching fluorescent-tagged dUTP nucleotides, FITC-dUTP, in a reaction catalyzed by terminal deoxynucleotidyl transferase and the amount of incorporated fluorescein was detected by flow cytometry. After incubation, treated and untreated cells were harvested, washed twice in PBS, and fixed in 1% (w/v) paraformaldehyde in PBS (pH 7.2) for 15 minutes on ice. After two more washing steps, cells were resuspended in ice-cold 70% ethanol and stored at −20°C until further use. According to the manufacturer's instructions, cells were washed twice in wash buffer, resuspended in 50 μL staining solution (10 μL reaction buffer, 0.75 μL terminal deoxynucleotidyl transferase, 8 μL FITC-dUTP, and 32 μL distilled water), and incubated for 1 hour at 37°C. After one more washing step, cells were resuspended in 1 mL propidium iodine/RNase solution and incubated in the dark for 30 minutes at room temperature. Subsequently, cell samples were analyzed by flow cytometry on a FACScan (Becton Dickinson, San Jose, CA).

Quantitative Real-time Reverse Transcription-PCR

RNA from ovarian cancer cell lines was isolated with the RNeasy Mini kit (Qiagen, Hilden, Germany) as described by the manufacturer. The cDNA synthesis was done after a DNase I treatment with 1 μg of total RNA using the Enhanced Avian HS reverse transcription-PCR kit (Sigma) according to the technical bulletin. Assay-on-Demand probes for the TaqMan real-time PCR system from Applied Biosystems (Foster City, CA) for the *DR4* and *DR5* genes and the internal housekeeping control-gene, *β-2-microglobulin* (*B2M*), were as follows: *DR4*, Hs00269491m1; *DR5*, Hs00366272m1; and *B2M*, Hs99999907_m1. Real-time PCR of 10 ng of the cDNA mixture of all probes in a volume of 20 μL were obtained in duplicates with the GeneAmp 5700 Sequence Detection System (Applied Biosystems) with the following cycle conditions: Initially, 50°C for 2 minutes and 95°C for

10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative expression (compared with a calibrator, a cDNA mixture of several cell lines) of all probes were calculated from the threshold cycles (C_t) obtained with the GeneAmp 5700 SDS Software Ver. 1.3 (Applied Biosystems) as follows: $2^{\wedge} - [(C_{t\ gene}$ mean of duplicated probes $- C_{t\ gene}$ mean of duplicated calibrator) $- (C_{t\ B2M}$ mean of duplicated probes $- C_{t\ B2M}$ mean of duplicated calibrator)].

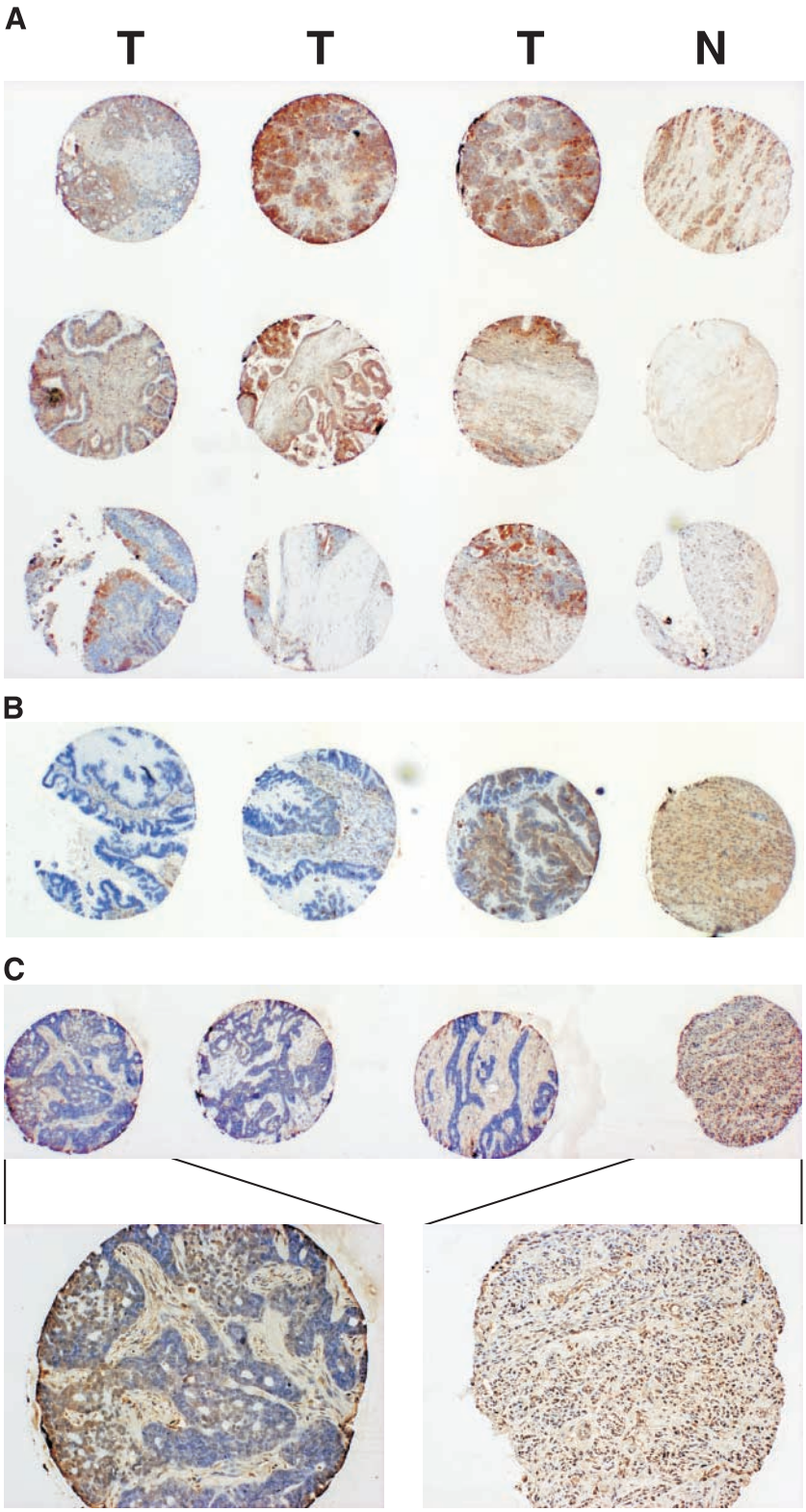


FIGURE 4. Immunohistochemical analysis of DR4 expression. Tissue microarray of tumor (*T*) and neighboring stromal (*N*) paraffin-embedded material. For each case, three tumor stamps were used. **A.** Expression in tumor tissue was not changed (>30%). **B.** Expression in tumor tissue was slightly down-regulated (10-30%). **C.** Expression in tumor tissue was down-regulated (<10%).

Identification of the DR4 CpG Island

In BLAST homology searches, the 5′ nucleotide sequence of *DR4* mRNA, corresponding to the first exon identified a bacterial artificial chromosome clone (AC100861) contained in the chromosome *8p22* human genome contig sequence NT_023666. The available sequence of this bacterial artificial chromosome was analyzed with a CpG prediction algorithm, according to the University of California Santa Cruz Genome Browser April 2003 draft of chromosome *8p21.2*, and depicted a CpG island spanning 1,018 bp (corresponding to nucleotides 22,902,527-229,033,544), which begins ~300 bp upstream of the translation start site of *DR4*.

Methylation-Specific PCR

One-microgram genomic DNA was treated with sodium bisulfite for 16 hours as described in ref. (24). Primers for bisulfite sequencing were designed to avoid any CpGs in their sequences using the MethPrimer software. The final PCR products were sequenced on an ABI 377 sequencer, followed by determination of methylated CpG islands, which were not altered by bisulfite. Methylation-specific primers for the *DR4* promoter were constructed using the MethPrimer software (25), amplifying a product of 114 bp. To further enhance the sensitivity and specificity of the methylation-specific PCR, we used nested methylation-specific primers after preamplification of the desired sequence with outer primers we primarily designed for bisulfite sequencing. Methylation-specific primers for the *DR5* promoter and appropriate PCR conditions were applied as published by van Noesel et al. (11) PCR conditions and primer sequences are available upon request.

DNA GeneChips Experiments

From two independent replicate experiments (mock and AzadC treated), total RNA was isolated and done with the HG-U133A and HG-U133B GeneChip (Affymetrix, Santa Clara, CA) according to the manufacturer's protocols. Results were analyzed using the GeneSpring 5.1 Software (Silicon Genetics, Redwood City, CA). Raw data including detection flags from Affymetrix were imported into the software, each chip normalized to the 50th percentile and each probe set was divided by the median of its measurements in all samples. After setting the analysis mode to Log of Ratio, the Cross-Gene Error Model was based on replicates. Reliable genes were filtered by control strength with the average of Base/Proportional (Cross-Gene Error Model, Gene Spring 5.1) value as minimum. Up-regulation was defined either as 3-fold change of expression and detection flags called at least once marginal/present after treatment or as change of detection flags from absent to marginal/present in both replicate experiments (AA→PP). Down-regulation was defined in the same way. Complementary of all probe sets representing all genes depicted in Table 1 were confirmed with reviewed mRNA sequences (if available) from the Reference Sequence collection (National Center of Biotechnology Information) using the probe match tool from the NETAFFX analysis center. In case of *DR4*, only 2 of 11 probes from the probe set on GeneChip HG-U133B (231775_at derived from an EST) are complementary to the reviewed *DR4* mRNA (NM_003844) and, therefore, values for this probe set were not used in Table 1.

Tumor DNA and Ovarian Tissue Microarray

For methylation studies, DNA preparations described previously were used (4). Briefly, ovarian tumor biopsies were obtained from patients undergoing initial staging or debulking laparotomy; before freezing, sample mass was reduced by dissecting normal tissue.

For immunohistochemical studies, paraffin material available from primary diagnosis was used. Patients gave informed consent according to the criteria used at the Medical University Vienna. Relevant clinical information was gathered and tissue samples and clinical data anonymized. A tissue array was assembled by taking core needle "biopsies" from specific locations in the preexisting paraffin-embedded tissue blocks and re-embedding them in an arrayed "master" block using techniques and an apparatus developed by Beecher Instruments, Micro-Array Technology. To achieve good representation of the tumor, three biopsies of tumor material and one of neighboring regular, mostly stromal ovarian tissue, were selected from each patient sample. Using this technology, each tissue sample is treated in an identical manner and the entire cohort is analyzed in one batch on a single slide. Reagent conditions are identical for each case, as are incubation times and temperatures, wash conditions, and antigen retrieval if necessary. A 4 to 5 μm paraffin section of this tissue microarray was deparaffinized and then the section was treated with 0.2% H_2O_2/PBS (pH 7.4) to quench endogenous peroxidase activity. After blocking with 2% normal goat serum for 30 minutes, section was incubated at 4°C overnight with primary antibody [rabbit polyclonal DR4 (H130), Santa Cruz Biotechnology, Inc., Santa Cruz, CA] in 1% normal goat serum. Horseradish peroxidase–linked donkey anti-rabbit secondary antibody (ECL, NA 934) was applied for 60 minutes followed by 4-minute incubation in diaminobenzidine substrate (DAKO Liquid DAB+). Section was counterstained in hematoxylin for 90 seconds and mounted under a coverslip.

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