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Effects of Androgen Ablation Therapy in TRAIL Death Ligand and Its Receptors Expression in Advanced Prostate Cancer

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Key Words
Prostate cancer · TRAIL · Androgen ablation therapy · Hormone-refractory prostate cancer

Abstract
Background: It is not known whether androgen ablation therapy (AAT) influences TRAIL death ligand and its receptors expression of prostate cancer (PCa) cells. ***Aim:*** To investigate whether hormonal therapy alters the expression of TRAIL death ligand and TRAIL receptors in patients with advanced PCa. ***Patients and Methods:*** 26 untreated and 20 AAT-treated advanced PCa patients were included in the study. The patients who received AAT were divided into two groups based on hormone sensitivity status. TRAIL ligand and receptor expression were determined by a conventional immunohistochemistry method. ***Results:*** TRAIL death ligand and TRAIL-R2 death receptor were upregulated in PCa patients who received AAT. Hormone-refractory PCa patients exhibited lower levels of TRAIL death receptor (TRAIL-R1 and TRAIL-R2) expression compared to hormone-sensitive PCa patients. ***Conclusions***: AAT alters TRAIL death ligand and its receptors expression in patients with PCa.

Introduction

The second most common cancer diagnosed in US males is prostate cancer (PCa). Estimates are that, in 2008, 186,320 cases of PCa were diagnosed in the USA and 28,660 men died of the disease [1]. The standard therapies for metastatic PCa block the action of androgens or remove the testicular androgens from the patient. These therapies include orchiectomy to physically lower testosterone levels and injections of LH-releasing hormone analogues to pharmacologically lower testosterone levels; treatment with antiandrogens, such as flutamide or bicalutamide, to block testosterone binding to the androgen receptor (AR), and maximal androgen blockade, in which antiandrogen treatment and androgen ablation therapy (AAT) are combined. Withdrawal of androgens through physical or chemical castration often leads to regression of the disease. However, although many tumors initially regress after such therapies, most of the tumors eventually begin to regrow at various rates in an androgen-refractory manner [2].

It is still unclear why many prostate tumors eventually become androgen-refractory. Prostate tumor cells appear to have several possible mechanisms by which

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they could become androgen-refractory. Progression of localized hormone-dependent PCa to metastatic hormone-refractory disease is also associated with dysregulation of normal apoptotic mechanisms [3–5]. The relationship between the apoptotic pathway and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising area of scientific interest for cancer researchers. TRAIL is a type 2 transmembrane protein that is cleaved by proteases to release a soluble form [6]. Although constitutively expressed in normal tissues, TRAIL preferentially induces apoptosis in tumor cells with minimal adverse effects on normal cells [6]. Therefore, TRAIL is an attractive candidate for development as a biologic agent for cancer therapy [7]. Collectively, five TRAIL receptors have been identified [8]. TRAIL-R1 and TRAIL-R2 act as transmembrane signaling death receptors with cytoplasmic death domains (DD) which respond to ligand binding and activate the extrinsic cell death pathway by facilitating interaction between the specific adapter protein (FAS-associated DD) and proapoptotic effector proteins (caspases 8 and 10) [8]. Two other membrane receptors, TRAIL-R3 and TRAIL-R4, are so-called decoy receptors because they can bind TRAIL but lack DD and are unable to induce cell death. Finally, osteoprotegerin, a regulator of osteoclastogenesis, has been reported to be a soluble receptor for TRAIL [9]. It has been suggested that the differences in the expression levels of death (TRAIL-R1, TRAIL-R2) versus decoy (TRAIL-R3, TRAIL-R4) receptors can determine the sensitivity of tumor cells to TRAIL-induced apoptosis [10]. It is not known whether AAT influences TRAIL death ligand and its receptors expression of PCa cells in patients with advanced PCa. Therefore, the aim of this study was to investigate whether hormonal therapy alters the expression of TRAIL death ligand and TRAIL receptors in patients with advanced PCa.

Patients and Methods

Clinical Assessment of Patients with PCa

26 untreated and 20 AAT- (LHRH agonist with or without antiandrogen) treated advanced PCa patients were included in the study. Advanced PCa patients possessed clinical and radiological evidence of metastatic disease. Transurethral resection procedures, utilized in order to alleviate infravesical obstruction, were employed to obtain prostate tissue samples from patients. Patients were followed up every 3 months in the urology department of our hospital. Patients who received AAT were divided into two groups based on hormone sensitivity status, namely hormone-sensitive (n = 10) and hormone-resistant (n = 10). The criteria for hormone sensitivity status of PCa were reported previously [11]. Androgen-dependent tumors were defined according to the criteria of symptom relief and more than 50% decrease of serum prostate-specific antigen (PSA) levels at diagnosis. Disease progression for APCA patients is defined as appearance of new lesion(s), and/or an increase of 25% of measurable metastases, and/or the appearance of new foci on a radionuclide bone scan, and/or three consecutive increases in PSA concentration at least 1 week apart in the presence of testosterone castrate level (<50 ng/ml) of metastatic patients. Patients were given a designated Gleason grading score based on the specimens obtained by transurethral resection of the prostate. Briefly, the Gleason grading system is based on a low-power microscopic description of the histologic architecture of cancer. A Gleason grade of 1–5 was assigned as a primary grade (pattern occupying the largest area of the specimen) and as a secondary grade (pattern occupying the second largest area). Adding the primary and secondary grades determined a Gleason score (2–10) [12].

Immunohistochemistry of TRAIL and Its Cognate Receptors

Serial sections were sliced from paraffin blocks and placed on slides. A deparaffinization procedure was employed by incubating slides at 58°C for 1.5 h. Following xylene treatment, serial ethyl alcohol washes were performed. Samples were heated in citrate buffer (pH 6) for antigen retrieval in a microwave oven at 750 W. After cooling samples to room temperature, slides were washed in Tris-buffered saline. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 10 min. Ultra V Protein Block (TA-060-UB, Lab Vision, USA) was used to inhibit non-specific binding on slides. Then sections were treated with primary antibodies for 1 h at room temperature. All primary antibodies were purchased from Alexis Biochemicals (Switzerland) and the following primary antibodies (1/300) were used for the immunohistochemical analysis of prostate specimens: monoclonal antibody to TRAIL (human (III6F) ALX-804-326-C100), monoclonal antibody to TRAIL-R1 (human (HS101) ALX-804-297A-C100), polyclonal antibody to TRAIL-R2 (ALX-210-743-C200), polyclonal antibody to TRAIL-R3 (human, ALX-210-744-C200), and monoclonal antibody to TRAIL-R4 (human (HS402) ALX-804-299A-C100). The sections were then treated with a biotinylated goat anti-polyvalent antibody (TP-060-BN, Lab Vision, USA) followed by streptavidin peroxidase treatment (TP-060-HR, Lab Vision, USA) for 20 min. Substrate-chromogen solution (DAB) was applied for 10 min to visualize peroxidase activity. Lastly, prostate sections were counterstained with hematoxylin for 5 min. Mainly membranous staining was detected using these primary antibodies. Specificity of these primary antibodies was previously confirmed by Alexis Biochemicals. As a negative control, specimens were immune-stained as described above in the absence of primary antibodies. No immune staining was detected when primary antibodies were not used.

Immunohistochemical Scoring of TRAIL and TRAIL Receptors

Slides of the specimens were analyzed by two independent pathologists (A.C. and I.C.B.) who had no prior knowledge of the clinical data. The immune-staining scores were assessed based on both the intensity and the marker distribution (percentage of positively stained epithelial cells) in prostate. The intensity was scored as follows: 0, negative; 1, weak; 2, moderate, and 3, strong

staining. The marker distribution was also scored as 0, <10%; 1, between 10 and 40%; 2, between 40 and 70%, and 3, >70% of the epithelial cells stained on the specimen. The final immune-staining score was obtained by adding scores of both the intensity and the marker distribution for a given patient.

Statistical Analysis

Statistical analyses between groups were made by the one-way ANOVA and Newman-Keuls multiple comparison test and Wilcoxon-matched pairs test, and p values <0.05 were considered significant; values are presented as the mean ± SD, median with 25th and 75th percentile scores. Data were analyzed using the Prism statistical package version 3 (GraphPad Prism, San Diego, Calif., USA).

Results

Immunohistochemical staining of prostate samples obtained from 26 untreated and 20 AAT-treated advanced PCa patients (total n = 46) is performed to reveal the expression profiles of TRAIL ligand (TRAIL-L) and TRAIL receptors. Figure 1 shows immunohistochemical staining samples of untreated (left panels) and AAT-treated (right panels) advanced PCa sections.

Microscopic analysis of prostate sections revealed higher levels of TRAIL-R4 decoy receptor expression in patients with untreated advanced PCa compared to levels of the other markers tested (fig. 2a). It is clear that TRAIL-R4 decoy receptor was the prominent TRAIL receptor expressed in untreated advanced PCa samples. Although expression levels of all TRAIL markers with the exception of TRAIL-R4 decoy receptor appear to increase in patients with AAT-treated advanced PCa, average TRAIL-R2 and TRAIL-L expression was highest in these patients. However, as shown in figure 2b, the differences between expression levels of all TRAIL markers did not reach statistical significance in these patients ($p > 0.05$). Comparison of untreated and AAT-treated advanced PCa patients indicated higher TRAIL-L and TRAIL-R2 death receptor expressions in PCa patients who received AAT ($p < 0.05$), as shown in figure 2c.

Patients who received AAT are divided into two groups based on hormone sensitivity status, namely hormone-sensitive (n = 10) and hormone-resistant (n = 10). As shown in figure 3a and b, the differences between expression levels of all TRAIL markers did not reach statistical significance in either hormone-sensitive or hormone-resistant PCa patients ($p > 0.05$). Hormone-refractory PCa patients exhibited significantly lower levels of TRAIL death receptor expression compared to hormone-sensitive PCa patients ($p < 0.05$) (fig. 3c).

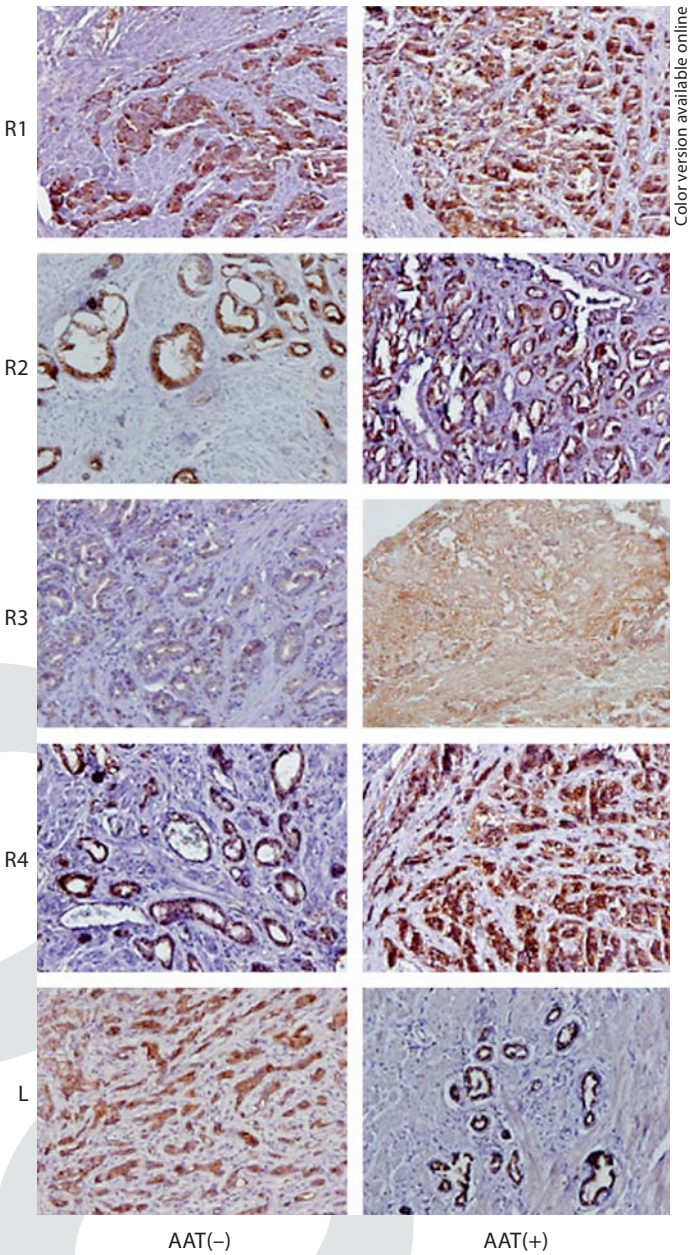

Fig. 1. Immunohistochemical staining of TRAIL and TRAIL receptors in 26 untreated (left) and 20 AAT-treated (right) advanced PCa patients. Duplicate staining samples for each antibody are shown. Reduced from ×200.

Discussion

PCa is dependent on androgen stimulation mediated by the AR, a member of the steroid hormone receptor family of ligand-dependent nuclear receptors. Most patients respond to standard androgen ablation therapies,

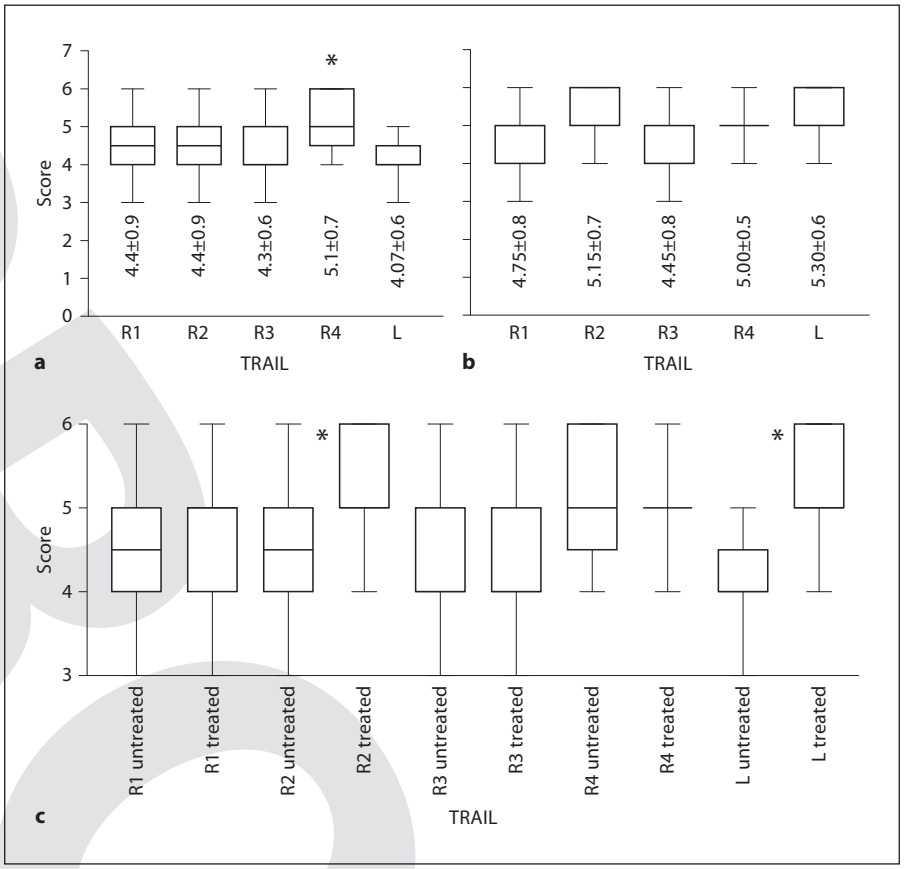

Fig. 2. Distinctive TRAIL ligand receptor expression patterns in untreated (**a**) and AAT-treated (**b**) advanced PCa patients. Upregulated TRAIL-L and TRAIL-R2 death receptor expressions in PCa patients who received ATT vs. untreated (**c**). Error bars represent ± SD. * $p < 0.05$.

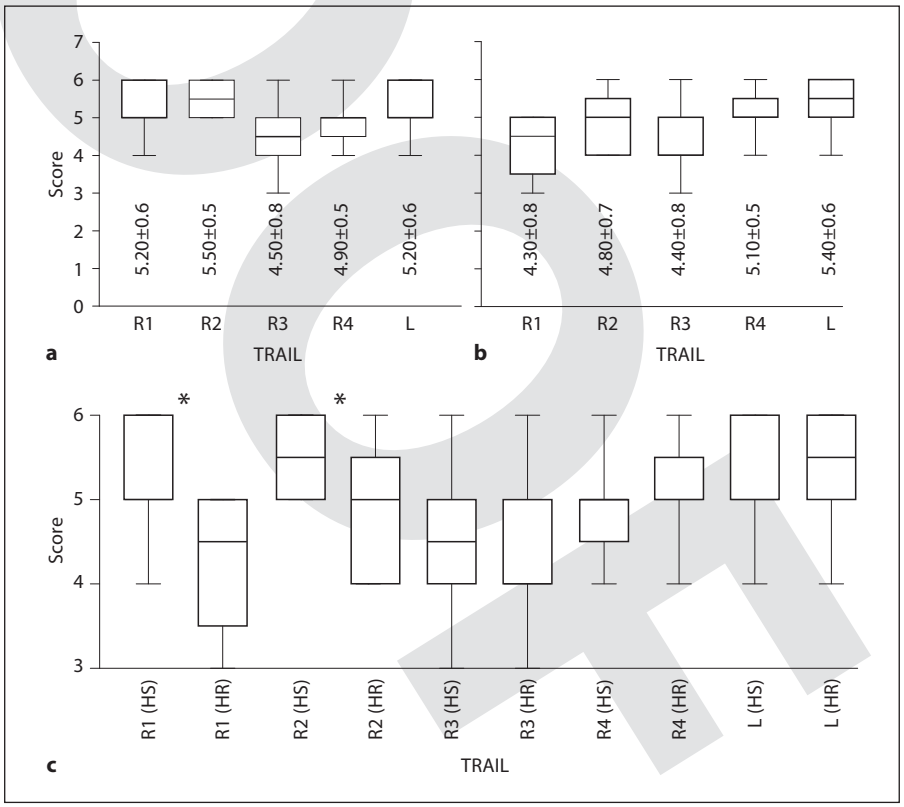

Fig. 3. Comparative analysis of the expression levels of TRAIL and its receptors in connection with hormone sensitivity status in patients who received AAT. Patients who received AAT were categorized into two groups based on hormone sensitivity status, namely hormone-sensitive, HS (n = 10) (**a**) and hormone-resistant, HR (n = 10) (**b**). Only TRAIL-R1 and TRAIL-R2 (death receptors) expression showed such a difference (**c**). Error bars represent ± SD (* $p < 0.05$).

but virtually all patients eventually relapse with disease that has been termed hormone-refractory or androgen-independent disease. The mechanism of the change in tumors from being androgen-responsive to androgen-unresponsive is generally explained by clonal selection, adaptation, an alternative pathway of signal transduction and AR involvement. Since androgen action is mediated by ARs, abnormalities in ARs are believed to play an important role in the progression of PCa [13, 14]. Defects in apoptotic signaling pathways are common in cancer cells. Impaired apoptosis may also enhance tumor progression and promote metastasis, and these result in an increase of cancer cell resistance to various forms of therapy [15, 16]. Progression of localized hormone-dependent PCa to metastatic, hormone-refractory disease is also associated with dysregulation of normal apoptotic mechanisms [3, 4]. Therefore, the current research emphasis is to identify agents that induce apoptosis in both androgen-responsive and androgen-refractory cells.

TRAIL is a member of the tumor necrosis factor (TNF) superfamily of cytokines that induces the extrinsic apoptotic pathway upon binding to its DD-containing transmembrane receptors. TRAIL binds to two cell-death-inducing (TRAIL-R1 and TRAIL-R2) and three decoy (TRAIL-R3, TRAIL-R4 and osteoprotegerin) receptors. Because the presence or absence of TRAIL decoy receptors were connected to the sensitivity of cancer cells to apoptotic ligands [10, 17–19], the modulation of TRAIL and TRAIL receptor expression might be essential for the progression of PCa [20]. The presence of high levels of TRAIL-R4 decoy receptor expression has recently played an important role in the development of a resistance mechanism to apoptotic ligands [17, 19]. A DcR2 (TRAIL-R4) siRNA approach followed by an Ad5hTRAIL infection might be of some use to overcome potential TRAIL resistance in patients with PCa [21]. Studies suggest that in many cancer cells only one of the two death-inducing TRAIL receptors is functional. These findings as well as the aim to avoid decoy receptor-mediated neutralization of TRAIL led to the development of receptor-specific TRAIL variants and agonistic antibodies. These molecules are predicted to be more potent than native TRAIL in vivo and may be suitable for targeted treatment of particular tumors [22]. Modulation of intracellular anti-apoptotic pathways represents another means of influencing TRAIL sensitivity. Nuclear factor-κB inhibition using a gene therapy approach (AdIKKbKA) or curcumin can sensitize hormone-refractory PCa cells to TRAIL-induced apoptosis [17, 23]. TRAIL-resistant tumors can be resensitized to TRAIL by a combination of TRAIL with chemotherapeutics or irradiation. Combination therapies enhance TRAIL-induced apoptosis through differential regulation of pro- and anti-apoptotic proteins. These effects, though beneficial in cancer, may also be detrimental to normal cells. The potential toxicities on human keratinocytes, endothelial cells and hepatocytes have been realized following studies using proteasome inhibitors and cycloheximide co-treatments [24–26]. How these and other therapeutics affect TRAIL receptor expression in primary, non-transformed cells is largely unknown. Thus, more focused preclinical and early clinical trials are required to elucidate the mechanism of TRAIL resistance of non-transformed cells and the safety profile of TRAIL when used in combination with other drugs [22].

The role of androgen regulation of TNF-related family-induced apoptosis is poorly understood. The androgen-responsive human PCa cell line LNCaP is resistant to TRAIL and TRAIL-mediated apoptosis in LNCaP is PI3K/Akt-dependent [17, 21, 27, 28]. Rokhlin et al. [28–30] demonstrated that LNCaP remained resistant to treatment with TRAIL after androgen deprivation, even in the presence of the PI3K/Akt pathway inhibitor wortmannin. This resistance was determined by failure to form the TRAIL-DISC (death-inducing signaling complex) and by decreased TRAIL-R1 and TRAIL-R2 levels after androgen deprivation; the capacity of TRAIL to induce DISC formation was completely restored in the presence of dihydrotestosterone (DHT). Their data suggest that TRAIL-DISC formation and sensitivity to TRAIL treatment are androgen-dependent in LNCaP [29]. It has also been shown that the apoptotic response to TNF-related family ligand treatment is regulated by DHT in a dose-dependent manner [30]. Vindrieux et al. [31] have recently investigated whether TRAIL and its receptors expression was targeted by androgens during the apoptotic cell death process in the hormone-sensitive ventral prostate. These authors showed that androgen deprivation associated with an apoptotic process resulted in a decrease in TRAIL-R4 mRNA and protein expression in the ventral prostate. Testosterone administration to castrated adult rats prevented the decrease in TRAIL-R4 mRNA and protein levels in the ventral prostate. No changes were observed in TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL mRNA and protein levels in prostate after castration. These results suggest that testosterone specifically controls TRAIL-R4 expression in the adult rat ventral prostate. We have previously shown

that benign and malignant PCa cells differentially display TRAIL and its receptors, and this profile was connected to prostate carcinogenesis. Analysis of prostate sections suggested that not only patients with benign prostate hyperplasia but also organ-confined PCa or advanced PCa displayed increased TRAIL-R4 receptor expression [20]. In addition, high TRAIL-R4 expression is correlated with high Gleason scores, PSA recurrence, and decreased survival in patients with PCa [32]. It is not known whether AAT influences TRAIL death ligand and its receptors expression of PCa cells in patients with advanced PCa. Therefore, the goal of this study was to investigate the importance of TRAIL-L/TRAIL receptor expression profiles in PCa patients who received AAT. Our analysis demonstrated that TRAIL-L and TRAIL-R2 death receptor are upregulated in advanced PCa patients who received AAT. But, after it progressed to the hormone-refractory stage following AAT, advanced PCa patients exhibited lower levels of TRAIL death receptor expression compared to hormone-sensitive PCa patients. This is not a surprise since TRAIL death receptor expression downregulation might be a way of escaping apoptosis. This finding suggests that hormone-refractory PCa cells arise as a subpopulation that has acquired the capacity to downregulate the expression of TRAIL death receptors. Combining recombinant TRAIL, such as agonistic antibodies and gene therapy, with existing or recently developed anticancer drugs should overcome this resistance and provide greater therapeutic benefits than the use of these compounds alone.

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