Original Article

Recurrence-Free Survival in Prostate Cancer Is Related to Increased Stromal TRAIL Expression

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BACKGROUND: TRAIL (tumor necrosis factor related apoptosis-inducing ligand) is involved in tumor immune surveillance and, thus, may be a potential cancer therapy. TRAIL expression in the tumor microenvironment has been shown to impact cancer survival in multiple tumor types, including ovarian cancer. We studied TRAIL expression and outcomes in patients with prostate cancer. **METHODS:** A tissue microarray (TMA) of 200 prostate cancer patients and benign prostate tissue controls was used to assess the epithelial and stromal protein expression of TRAIL, death receptors (DR4 and DR5), decoy receptors (DcR1 and DcR2), and the FLICE inhibitory protein ($FLIP_L$). We correlated these expression patterns with clinicopathological parameters and determined its impact on recurrence-free survival. **RESULTS:** Nearly all (99.5%) prostate cancer tissues examined displayed either decreased expression of pro-apoptotic TRAIL receptors, increased $FLIP_L$ expression, or both. We observed elevated death receptor, decoy receptor, $FLIP_L$, and epithelial TRAIL expression in prostate cancer epithelium. TRAIL expression in the stromal tumor microenvironment surrounding the prostate cancer was markedly lower. Elevated TRAIL expression in the tumor microenvironment was also significantly associated with increased recurrence-free survival ($P = .014$), after controlling for other prognostic markers. In contrast, epithelial expression of TRAIL did not have an effect on overall survival. **CONCLUSIONS:** Expression of the components of the pro-apoptotic TRAIL pathway is altered in prostate cancer. Moreover, TRAIL expression in the tumor microenvironment may affect recurrence-free survival rate of prostate cancer patients. Consequently, these results may be useful in devising future therapeutic strategies targeting the TRAIL pathway in prostate cancer. ***Cancer*** **2011;117:1172–82.** *© 2010 American Cancer Society.*

KEYWORDS: Prostate cancer, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), death receptors, recurrence-free survival.

With approximately 200,000 deaths per year worldwide, prostate cancer is one of the most frequent causes of cancer mortality among men. Prostate cancer develops by an uncontrolled expansion of the prostate epithelial cells.[1] Epithelial atrophy and high-grade intraepithelial neoplasia (PIN) are known precursors to prostate cancer.[2] Well-established risk factors for prostate cancer include age, race, and family history.[2,3] Genetic and epigenetic alterations account for the malignant transformation and progression of prostate cancer.[4-6] Most prostate cancers display mutations in p53 and androgen receptor (AR).[7] Additional genetic alterations and dysregulations typically affect a variety of cellular functions, including transcription (E2F4), telomerases, kinases (MAPK, PIM1), phosphatases (PTEN), adhesion proteins (E-cadherin), proteases (hepsin), metabolizing genes (AMACR), and even nonprotein coding genes ($DD3^{PCA3}$).[7]

Despite best available treatment, 30%-40% of patients may experience recurrence.[8] Androgen deprivation constitutes a highly effective form of prostate cancer treatment. The therapeutic challenge remains the development of androgen-independence, thus resulting in a need for chemotherapeutic drugs. Recently, docetaxel chemotherapy has been established as first-line therapy in androgen-independent prostate cancer.[9] However, patients with advanced disease eventually develop resistance to docetaxel and are left with few effective alternatives.[10]

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Table 1. Clinicohistopathologic Characteristics of Prostate Cancer Patients

	Patients (%)	Regular Receptor Expression n/N[a]	$FLIP_L$ n/N	Epithelial TRAIL n/N	Stromal TRAIL n/N
Benign histology		9/199 (4.5%)	123/199 (61.8%)	101/199 (50.8%)	120/199 (60.3%)
Tumor histology		53/199 (26.6%)	178/199 (89.4%)	173/199 (86.9%)	57/199 (28.6%)
Tumor stage					
<T3	75.2	21/105 (20%)	69/105 (65.7%)	65/105 (61.9%)	25/105 (23.8%)
≥T3	24.8	6/105 (5.7%)	21/105 (20%)	19/105 (18.1%)	5/105 (4.8%)
Gleason score					
<7	43.8	14/105 (13.3%)	40/105 (38.1%)	38/105 (36.2%)	12/105 (11.4%)
≥7	56.2	13/105 (12.4%)	50/105 (47.6%)	46/105 (43.8%)	18/105 (17.1%)
Age at diagnosis, y					
<60	63.8	20/105 (19%)	60/105 (57.1%)	55/105 (52.4%)	21/105 (20%)
≥60	36.2	7/105 (6.7%)	30/105 (28.6%)	29/105 (27.6%)	9/105 (8.6%)

$FLIP_L$, long form of FLICE-like inhibitory protein; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.
[a] Positively stained/total no. of patients analyzed.

TNF-related apoptosis inducing ligand (TRAIL) is known to be a stimulator of apoptosis in transformed cells and, thus, a potential therapy. Its clinical significance derives from its ability to trigger cell death in tumor cells while simultaneously having no effect on normal cells.[11] So far, 5 TRAIL receptors have been identified in humans, including functional death receptors (DR4 & DR5), decoy receptors (DcR1 & DcR2), and osteoprotegerin (OPG). An intracellular adaptor protein (FADD) further relays the pro-apoptotic signal to caspase 8, leading to the formation of the death inducing signaling complex (DISC), activation of effector caspases, and, consequently, to apoptotic cell death via the extrinsic pathway.[12-15] FLICE Inhibitory Protein (FLIP) inhibits the activation of caspase-8 by preventing the assembly of DISC.[13] FLIP, thus, might be involved in a resistance mechanism to TRAIL-induced apoptosis.[16,17] In addition, because of its expression on the surface of cytotoxic T cells and natural killer cells, TRAIL is also being extensively studied in cancer immune surveillance.[18]

Tumor cells have an ability to escape host innate and adaptive immune responses through various escape mechanisms.[19] In terms of TRAIL resistance, these include mutation or downregulation of death receptors, methylation of caspase-8 encoding genes, and overexpression of FLIP, thus potentially disabling immunosurveillance regulating mechanisms by host cytotoxic T cells.[20-22] Our previous work showed that DR4 is functionally silenced in a significant percentage of ovarian cancer patients, and TRAIL expression in the ovarian tumor microenvironment is linked with overall survival.[23,24] To study the TRAIL pathway in prostate cancer, we determined the expression pattern of TRAIL, death receptors DR4 and DR5, decoy receptors DcR1 and DcR2, and the apoptosis inhibiting protein $FLIP_L$ in the prostate cancer tissue of 200 patients. To assess the prognostic role of the TRAIL pathway in prostate cancer, we also determined the effect of expression of the individual components on recurrence-free survival.

MATERIALS AND METHODS

Prostate Tissue Microarray

This study was approved by the Institutional Review Board of the Dana-Farber Cancer Institute (DFCI). Four arrayed panels of prostate cancer tissue from 200 patients treated at a single institution (DFCI) were used to assess the epithelial and stromal protein expression of TRAIL, death receptors (DR4 and DR5), decoy receptors (DcR1 and DcR2), and the long form of the FLICE inhibitory protein ($FLIP_L$). These were then compared with benign prostate tissue. Three biopsies of tumor tissue and 1 to 2 biopsies of benign tissue from the same patient sample were taken. Clinicohistopathologic characteristics of the prostate cancer patients, including age at diagnosis, tumor stage, and Gleason score, are summarized in Table 1.

Immunohistochemistry

An arrayed panel of prostate cancer tissues was prepared using techniques and apparatus developed by Beecher Instruments Micro-Array Technology (Sun Prairie, WI). To achieve good representation of the tumor, 3 biopsies of tumor material were selected from each patient sample. Four-5 μm thick tissue microarray sections were deparaffinized by heating at 60C and subsequently rehydrated in

xylene and graded alcohols. Antigen retrieval was performed with DEPP-9 epitope retrieval solution (EB-depp9-250, eubio, Vienna, Austria), followed by treatment with 0.3% H_2O_2 in PBS (pH 7.4) to quench endogenous peroxidase activity. After blocking with 10% secondary antibody host serum for 10 minutes, the sections were incubated in primary antibodies [Rabbit polyclonal DR4 (H-130) sc-7863 (1:600 dilution); Goat polyclonal DR5 (C-20) sc-7191 (1:300 dilution); Goat polyclonal $FLIP_L$ (C-19) sc-7108, raised against a peptide mapping at the c-terminus of FLIP-long of human origin (absent in FLIP-small[25]) (1:300 dilution); Goat polyclonal TRAIL (K-18) sc-6079 (1:300 dilution), Santa Cruz Biotechnology, Inc, Santa Cruz, CA; Goat polyclonal DcR1 210-744 (1:2000 dilution), Alexis Biochemicals, Lausen, Switzerland; Rabbit polyclonal DcR2 ab2019 (1:150 dilution); Rabbit polyclonal Fibroblast Activation Protein (FAP alpha) ab53066 (1:200 dilution), Abcam, Cambridge, UK] for 1 hour at room temperature. Primary antibody dilutions were made in 10% secondary antibody host serum. The sections, after 2 PBS washes, were incubated in respective biotinylated secondary antibodies [Biotinylated anti-rabbit IgG (BA-1000); Biotinylated anti-goat IgG (BA-5000); Vector Laboratories, Inc. Burlingame, CA], diluted 1:200 in 10% serum for 30 minutes at room temperature, followed by 45 minute incubation in StreptABComplex/HRP (K0377 Dako, Denmark). The sections were again washed twice with PBS and incubated in Dako Liquid DAB + Substrate-Chromogen System (K3468 Dako Corporation, Carpinteria, CA) until the development of brown color. This was followed by counterstaining with Meyer's hematoxylin, dehydration, and mounting using Eukitt medium. The cells were counted and grouped according to percentage of positive cells as $<10\%$, 10%-30%, and $>30\%$. Intensity of staining was determined on a scale of 1 to 3, with 1 for weak, 2 for moderate, and 3 for strong. The staining analysis was performed by 3 independent investigators.

Statistical Analysis

For TRAIL and $FLIP_L$, staining was considered positive if greater than 10% of the cells showed moderate or strong staining; for TRAIL receptors, moderate or strong staining in greater than 30% cells was deemed positive, as described previously.[24] Regular (or positive) receptor expression occurred when either both death receptors were strongly stained or 1 was strong and the other moderately stained.[24] For statistical analysis, the following were used as binary variables: histology (tumor vs benign), stage ($<$T3 vs $>$T3), Gleason score (<7 vs ≥ 7), and age at diagnosis (<60 vs ≥ 60).

Comparison of TRAIL components' expression between tumor, PIN, and benign samples was made using paired *t*-test, and coexpression was determined by Spearman correlation. To analyze the dependence of the immunohistochemical variables on clinical variables, Kendall's tau-b correlation coefficients were computed. The date of disease recurrence was defined as the date of the first of 2 consecutive PSA values ≥ 0.2 ng/mL (determined at least 60 days after radical prostatectomy); or the date treatment was given when the PSA was less than 0.2 ng/mL, but greater than 0. Because there were only 3 deaths observed during follow-up, a combined endpoint of recurrence or death was analyzed. Recurrence-free survival was defined as the duration of time (in years) from radical prostatectomy to recurrence or death, and was censored at the date of the last available PSA value in case of no recurrence. Survival analysis was performed in 105 patients who were followedup for a median of 5.2 years (25th percentile: 3.3 years, 75th percentile: 8.4 years). Survival curves were depicted by the Kaplan-Meier method.[26] Crude hazard ratios (HR) and 95% confidence intervals (95%CI) were estimated by univariate Cox regression analyses[27] of recurrence-free survival on stage, grade, and the immunohistochemical variables. Because there were only 25 events observed, adjusted HRs for the immunohistochemical variables were obtained by bivariable Cox regression analyses adjusting only for Gleason score. *P* values of .05 or less were considered statistically significant. The SAS System V9.2 (2008 SAS Institute Inc., Cary, NC) was used for statistical analysis.

RESULTS

TRAIL expression is diminished in the microenvironment of prostate cancer

As a first step, we determined the protein expression of DR4, DR5, DcR1, DcR2, $FLIP_L$, and TRAIL (Figs. 1A and 1B) in a prostate tissue microarray using immunohistochemistry. Staining intensities of individual proteins in tumor tissues were compared with those within normal prostate and prostatic intraepithelial neoplasia (PIN). We aimed to contrast the expression of TRAIL pathway components in prostate cancer epithelium with its surrounding stromal tissue (composed of stromal fibroblasts and infiltrating immune cells). We observed significant differences between tumor tissues and corresponding normal controls when directly comparing immunohistochemical

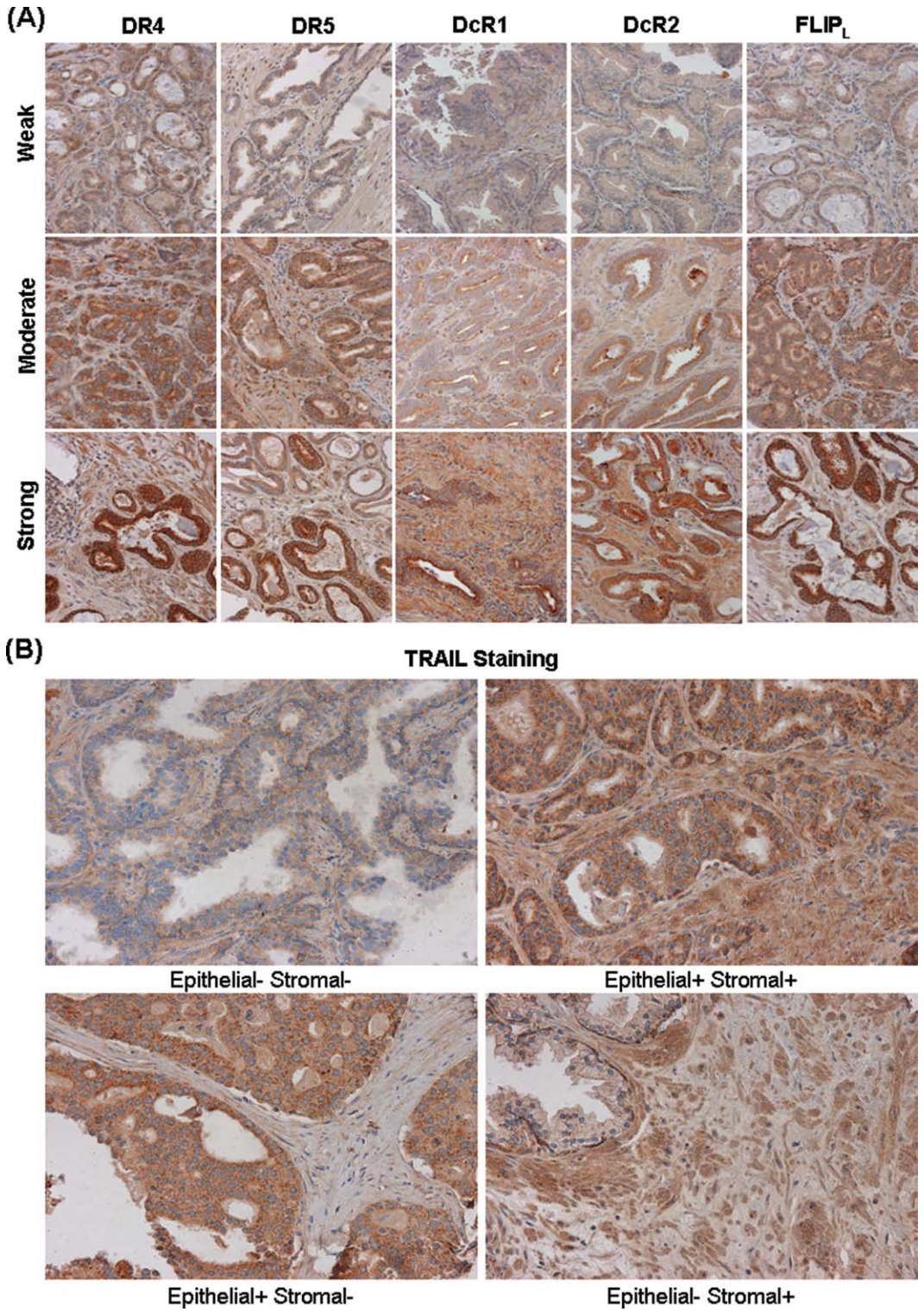

Figure 1. Immunohistochemistry of TRAIL pathway components. (A) DR4, DR5, DcR1, DcR2, and FLIP$_L$ expression in the prostate tissue microarrays. The immunohistochemical staining intensities were determined as weak,[1] moderate,[2] and strong,[3] (B) the specificity of TRAIL expression in the epithelium and stroma of the prostate tissue. (Magnification, 20x)

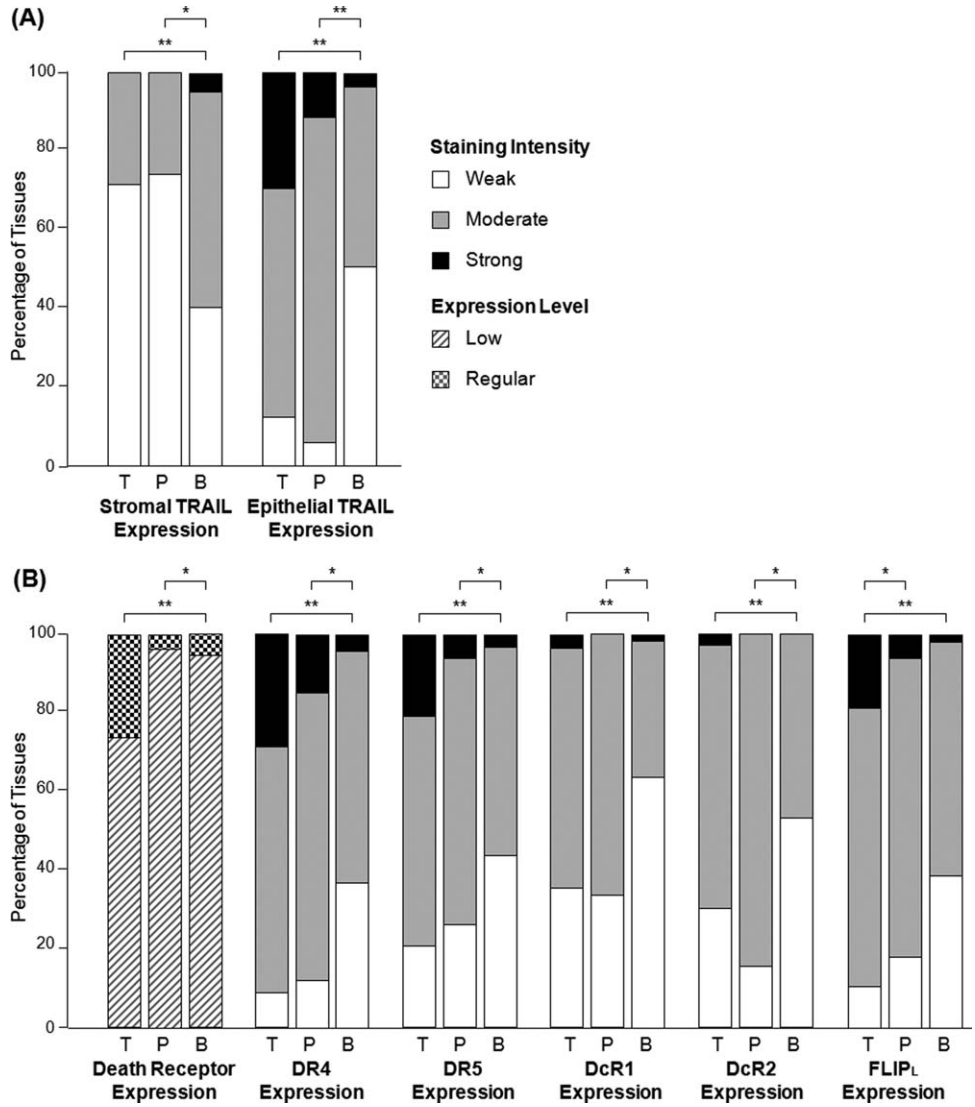

Figure 2. Differences in expression between prostate tumors, PINs, and benign tissue. (A) comparison of stromal and epithelial TRAIL expression, (B) comparison of TRAIL receptors and $FLIP_L$ expression between the tumor (T), PIN (P), and benign (B) prostate tissues. *$P < .05$; **$P < .001$.

TRAIL expression in epithelial tumor cells and surrounding stromal cells (Fig. 2A). In normal prostate tissue, a higher percentage of TRAIL expression in stromal cells (120/199, 60.3%) was noted, compared with prostate epithelium (101/199, 50.8%). In contrast to this finding, stromal TRAIL expression was significantly lower (57/199, 28.6%) among prostate cancers, whereas epithelial expression was increased (173/199, 86.9%, Table 1). Consequently, epithelial TRAIL was expressed more prominently in tumors and PINs when compared with normal tissue. Conversely, stromal TRAIL was significantly higher in benign tissues than in prostate cancer and PIN, respectively ($P < .001$, $P = .032$, Fig. 2A). These findings prompted us to further evaluate the possibility of impaired TRAIL signaling in the course of prostate cancer pathogenesis. Individual specificities of epithelial and stromal TRAIL staining are shown in Figure 1B.

Death receptors, decoy receptors, and $FLIP_L$ are highly expressed in prostate cancer

To assess the expression of both death receptors in a single variable, we further categorized the immunohistochemical readings according to Horak et al.[24] In short, positive (or regular) expression was assumed when either both death receptors were strongly positive or at least 1 strongly and the other moderately positive. All other

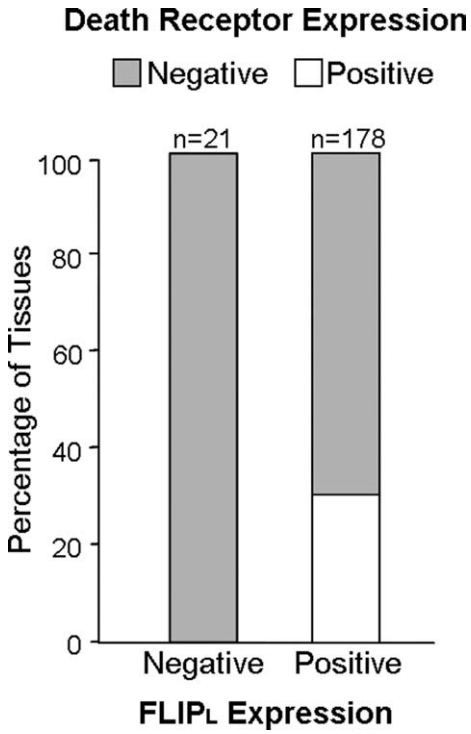

Figure 3. Death receptor expression correlates with $FLIP_L$ in the prostate tumor tissues. The subset of patients with negative $FLIP_L$ expression also has missing death receptor expression (n=21/21), compared with a percentage of $FLIP_L$ expressing patients with positive (regular) death receptor expression (n=53/178). All patients having positive death receptor expression also have positive $FLIP_L$ expression (n = 53/53; R = 0.731, $P < .001$).

combinations were regarded as negative or missing. By using these criteria, we could observe regular epithelial receptor expression in up to 26.6% of the tumor tissues, compared with only 4.5% in the benign samples, evidencing an increase of proapoptotic receptors in prostate tumors ($P < .001$, Fig. 2B). DcR1 was expressed in 64.3% of the tumors and 35.7% of the benign tissues ($P < .001$), whereas DcR2 expression was observed in 70.3% of the tumors and 29.6% of the benign samples ($P < .001$, Fig. 2B). $FLIP_L$ expression in the tumor tissues and normal samples proved disparate as well, registering 89.4% and 61.8%, respectively ($P < .001$, Fig. 2B and Table 1). Physiologically, this could be a result of counterregulation to the increased death receptor expression in prostate cancers. In line with this hypothesis is the observation that the PIN samples consistently showed an intermediate expression pattern for all the TRAIL components except decoy receptors, which had a similar expression pattern in the tumor and PIN tissues (Figs. 2A and 2B). To further link together our observations of differential expression of TRAIL-related genes in prostate cancers and normal tissues, we also assessed their degree of correlation.

$FLIP_L$ expression correlates with regular expression of death receptors in prostate cancer

Tumor cells can escape TRAIL-induced apoptosis by either downregulating death receptors or overexpressing $FLIP_L$. To determine whether these 2 events are independent of each other in prostate cancers or if they occur simultaneously, we investigated the relationship between TRAIL death receptor and $FLIP_L$ expression. A positive correlation was found between death receptor and $FLIP_L$ expression (R = 0.731, $P < .001$, Fig. 3), which suggests that cancer cells might evade TRAIL-induced apoptosis (brought about by increased death receptor expression) by increasing the intracellular levels of the anti-apoptotic protein $FLIP_L$. This correlation highlights the role of $FLIP_L$ in the inhibition of the apoptotic pathway in tumors with elevated death receptor expression (Fig. 3). According to this hypothesis, $FLIP_L$ was also increased in tumors expressing high epithelial TRAIL levels (R = 0.596, $P < .001$). This coregulation was not observed for stromal TRAIL expression, and was even reversed (R = −0.164, $P = .021$). Consequently, high epithelial TRAIL expression also correlated with increased death receptor expression (R = 0.713, $P < .001$). Notably, almost all (199/200, 99.5%) of the tumors in this microarray showed alterations in the TRAIL pathway, manifesting as elevated $FLIP_L$ expression (53/199, 26.6%), low expression of death receptors (21/199, 10.6%), or both (125/199, 62.8%). This fact suggests that evasion from TRAIL-induced apoptosis in prostate cancer may be an essential element of tumor development.

TRAIL receptor expression decreases with increase in Gleason score and age

We investigated the impact of TRAIL pathway aberrations in prostate cancer using several important clinical parameters, including age at diagnosis, tumor stage, and Gleason score. Clinical data were fully available and subsequently analyzed in a subset of 105 patients.

Gleason score is a measure of prostate cancer differentiation and a well-defined prognostic factor. In our analysis, prostate tumors with a Gleason score of 7 or more showed a significant reduction in DR4 expression, compared with the tumors with low Gleason score (R = −0.225, $P < .001$, Fig. 4), correlating more aggressive and undifferentiated cancers with low expression of apoptotic death receptors.

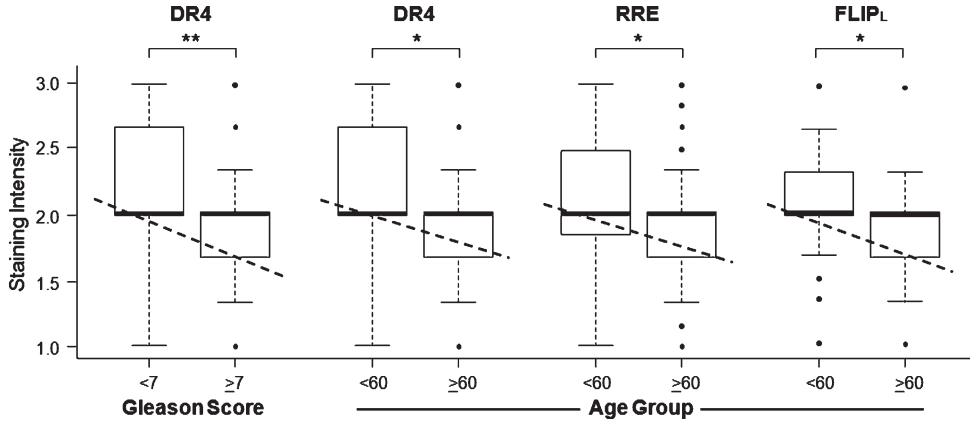

Figure 4. Correlation of death receptor and $FLIP_L$ expression with clinical parameters. Box plots showing quartiles of staining intensity comparing DR4 expression between patients with Gleason score of <7 and high risk patients with Gleason score ≥7; as well as comparing DR4, combined death receptor (RRE), and $FLIP_L$ expression between age groups of <60 and ≥60.

We also defined a weak, but statistically significant, effect of age on regular receptor expression. Interestingly, in the cancers of patients equal to or older than 60 years at diagnosis, we found lower overall TRAIL death receptor expression ($R = -0.125$, $P = .041$, Fig. 4). Individual analysis yielded a significant contribution of DR4 to the death receptor downregulation with increasing age, which would prefigure a less favorable outcome in elderly prostate cancer patients ($R = -0.142$, $P = .028$, Fig. 4).

In contrast, we also found diminished $FLIP_L$ expression in tumors from older patients ($R = -0.132$, $P = .042$, Fig. 4), also suggesting a reduced inhibition of TRAIL-induced apoptosis in this population. Decoy receptors did not show any significant correlation with the defined clinical parameters, although their expression was significantly increased in tumor samples.

$FLIP_L$, death receptor, and decoy receptor expression do not influence prostate cancer survival

Finally, we determined the effect of known prognostic variables—such as tumor stage and Gleason score, along with the expression pattern of TRAIL death receptors, decoy receptors, $FLIP_L$, and stromal and epithelial TRAIL—on recurrence-free survival of prostate cancer patients. Kaplan-Meier analysis showed, as expected, higher risk of recurrence for patients with T3 stage or higher versus less than T3 (HR 3.6, 95% CI, 1.6 to 7.9, $P = .001$), and patients of Gleason score 7 or higher versus less than 7 (HR 4.8, 95% CI, 1.6 to 14.3, $P = .004$, Figs. 5A and 5B). After adjusting for Gleason score, no significant correlation between $FLIP_L$ expression and risk of recurrence could be observed (HR 0.59, 95% CI, 0.22 to 1.55, $P = .282$). Individual DR4 and DR5 expression showed no effect on recurrence-free survival (HR 0.62, 95% CI, 0.31 to 1.35, $P = .249$ and HR 0.66, 95% CI, 0.36 to 1.24, $P = .199$, respectively). However, loss of regular receptor expression (DR4 and DR5 combined) was associated with a trend toward lower recurrence-free survival, both unadjusted (HR 0.35, 95% CI, 0.10 to 1.17, $P = .088$) and adjusted for Gleason (HR 0.31, 95% CI, 0.09 to 1.05, $P = .059$, Fig. 5C). Although this effect did not reach statistical significance, an additional cohort and/or a longer follow-up period may help to clarify this observation. DcR1 and DcR2 expression were not associated with recurrence-free survival of prostate cancer patients, both unadjusted (HR 0.77, 95% CI, 0.35 to 1.70, $P = .515$ and HR 1.90, 95% CI, 0.71 to 5.08, $P = .199$, respectively) and adjusted for Gleason (HR 0.991, 95% CI, 0.44 to 2.24, $P = .983$ and HR 2.694, 95% CI, 0.99 to 7.33, $P = .052$, respectively).

Stromal TRAIL expression influences recurrence-free survival in prostate cancer

We hypothesized that TRAIL expression might affect survival of prostate cancer patients similarly to what we have seen in ovarian cancer.[24] The effect of epithelial TRAIL on recurrence-free survival (unadjusted: HR 0.65, 95% CI, 0.28 to 1.52, $P = .324$; adjusted for Gleason: HR 0.69, 95% CI, 0.29 to 1.63, $P = .398$) did not reach statistical significance. However, we found that higher TRAIL expression in the prostate tumor microenvironment was associated with better recurrence-free survival. Furthermore, the effect of stromal TRAIL expression on survival was independent of Gleason score in a multivariate analysis (HR 0.22, 95% CI, 0.06 to 0.74, $P = .014$,

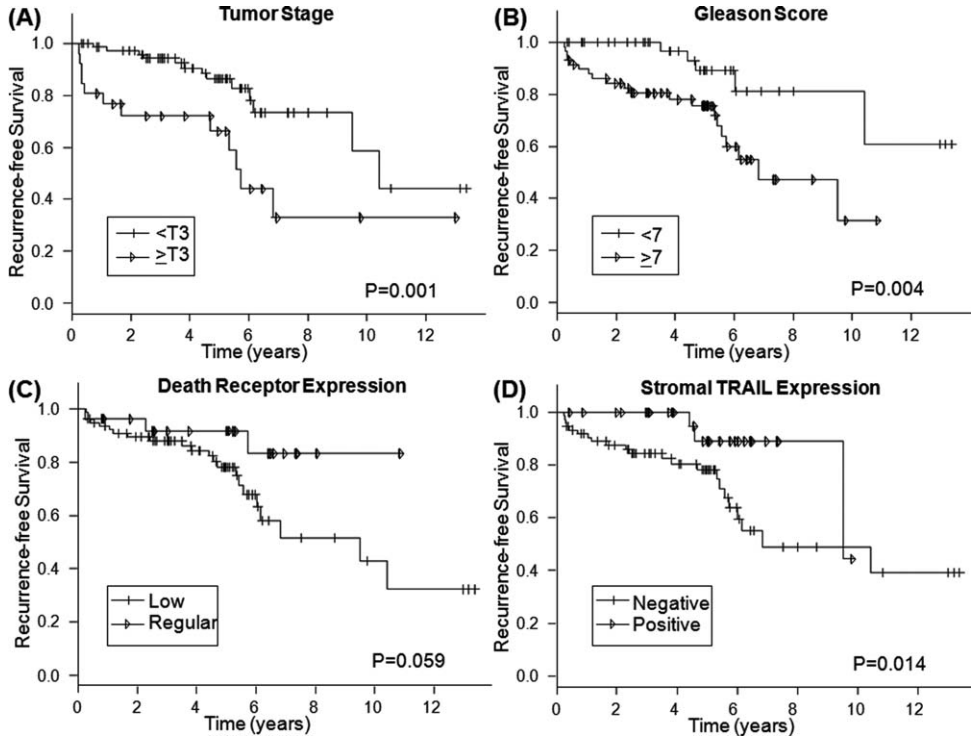

Figure 5. Recurrence-free survival in prostate cancer patients. Recurrence-free survival was displayed as Kaplan-Meier curves and stratified to (A) tumor stage, (B) Gleason score, (C) death receptor expression, and (D) stromal TRAIL expression. *P*-values are from univariable Cox regression (A and B) or from bivariable Cox regression adjusted for Gleason score (C and D).

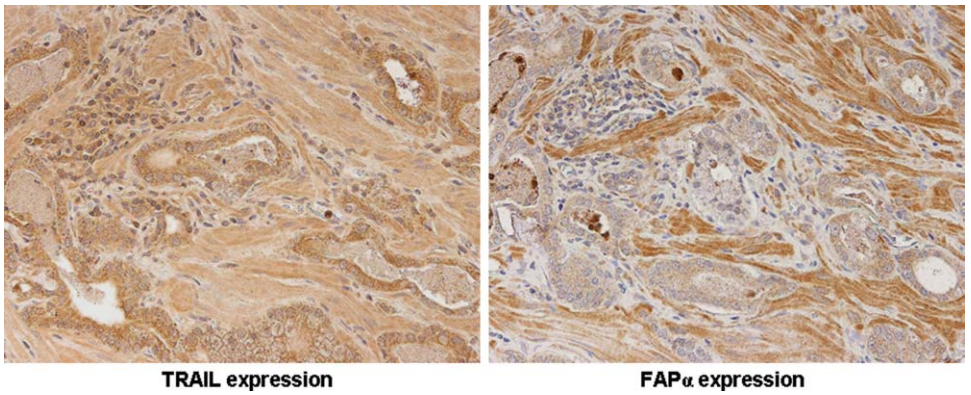

Figure 6. Comparison of FAP alpha and TRAIL staining in prostate cancer microenvironment. Expression pattern of the fibroblast activation protein (FAP alpha) correlated significantly with stromal TRAIL expression and could be attributed to stromal fibroblasts.

Figure 5D). This result suggests the significance of TRAIL pathway function in prostate cancer and defines prognostic value of TRAIL expression in the prostate tumor microenvironment. To investigate whether TRAIL expression in the tumor microenvironment can be attributed to cancer associated stromal fibroblasts (or rather tumor-infiltrating immune cells), we performed stainings with fibroblast activation protein (FAP alpha) antibody. Stroma of human prostate tumors is known to be composed of fibroblasts and myofibroblasts.[30] FAP alpha is selectively expressed in reactive stromal fibroblasts of epithelial cancers and granulation tissue of healing wounds. We could observe strong FAP alpha expression pattern in the tumor microenvironment, which correlated significantly with stromal TRAIL expression (Fig. 6, $R = 0.51$, $P < .001$).

DISCUSSION

Prostate cancer is the most common cancer in men and the second leading cause of cancer death in men after lung cancer. Although most patients with advanced cancer will respond to androgen deprivation therapy, most of these patients will ultimately progress to a so-called castration resistant state. Currently, therapeutic options for such patients are limited.[28] Human recombinant TRAIL or agonistic antibodies against its death receptors might represent a novel class of therapies for castration-resistant prostate cancer. However, to devise a TRAIL-based targeted therapy, we have an urgent need for markers and determinants of successful TRAIL-directed treatment in prostate cancer patients.

To address this issue, we used a prostate tissue microarray (TMA) to assess the expression of TRAIL, its apoptosis inducing receptors DR4 and DR5, the decoy receptors DcR1 and DcR2, and the inhibitory protein $FLIP_L$ in prostate cancer, prostatic intraepithelial neoplasia, and benign prostatic tissue. The advantages of a TMA are that it not only provides identical conditions for immunohistochemistry, but also allows for comparisons of a considerable number of tumors and benign tissues on the same slide.[29]

First, we found a significant increase in the expression of epithelial TRAIL and a loss of TRAIL expression from the tumor microenvironment in prostate cancers, compared with normal tissue. This observation is not completely analogous to our earlier observations in ovarian cancer, where we saw a uniform increase of stromal TRAIL expression in tumors.[24] Reasons for this difference may lie in the different tissues of origin and thus differing pathogeneses of these 2 cancer entities as well as in differential stromal-epithelial interactions of tumor cells. Nevertheless, although prostate cancers expressed significantly lower levels of stromal TRAIL than the benign controls, we could still observe a marked impact of stromal TRAIL levels on prostate cancer survival, which is consistent with the results obtained in ovarian cancers. Prostate cancer-associated stroma is largely composed of fibroblasts and myofibroblasts, in addition to smooth muscle cells, macrophages, endothelium, neurons, etc. In high-grade prostate tumors fibroblasts and myofibroblasts prevail,[30] suggesting only minor contributions of other stromal components to the pattern observed in our study.

Most importantly, the effect of stromal TRAIL on recurrence-free survival was independent of other prognostic factors. This fact not only confirms the findings of our previous work, but also highlights a possibly larger role of TRAIL in cancer pathogenesis. The microenvironment of prostate cancer epithelia is known to undergo alterations with advancing age to promote prostate carcinogenesis.[31,32] It has been shown that androgen-responsive stromal fibroblasts can promote invasiveness of prostate cancer in vivo and mediate hormonal carcinogenesis independently of epithelial cells.[33] Microarray profiling data from stromal tissue of aging prostates show multiple dysregulations concerning several stroma-derived factors, including cell surface molecules, secreted soluble factors, and matrix proteins.[34,35] In relation to our data, we were able to identify TRAIL as another important stromal tumor suppressor factor in prostate carcinogenesis, as its stromal levels seem to decline in the course of malignant transformation. Moreover, expression of stromal TRAIL in prostate cancer correlates with recurrence-free survival, thus pointing toward TRAIL-induced apoptosis as an important, independent prognostic factor in prostate cancer.

Second, we described elevated death receptor, decoy receptor, and epithelial TRAIL expression in tumors in comparison to the benign tissues. High death receptor expression suggests susceptibility to apoptosis and, thus, decreased tumorigenesis. At the same time, $FLIP_L$ expression in the tumor tissues was markedly higher and correlated significantly with death receptor and epithelial TRAIL expression. Consequently, as $FLIP_L$ is well known to inhibit caspase-8 activation and consecutive apoptotic pathways,[36] $FLIP_L$ overexpression in prostate cancers may lead to an inhibition of apoptosis induction, despite high death receptor or TRAIL levels. Following this reasoning, the opposite does not necessarily have to be true. Low death receptor expression may suffice to inhibit any TRAIL-induced apoptosis, despite low $FLIP_L$.

Molecular mechanisms that contribute to TRAIL, TRAIL receptors, and $FLIP_L$ overexpression in prostate cancer epithelium are currently under investigation and subject to multiple hypotheses. Transcriptional regulation of DR5 has been studied extensively and several factors have been identified, including p53,[37,38] YY1,[20] and NF-kappa B.[39] FLIP is also regulated by a plethora of transcriptional regulators, such as p53,[40] NF-kappa B, Foxo3a, and c-Myc.[41-44] The individual characteristics of transcriptional (or possibly even posttranscriptional) mechanisms that might be responsible for TRAIL pathway component overexpression in prostate cancer will have to be determined in further experiments.

Third, available clinical data offer some invaluable additional insights into the interaction between

expression of TRAIL pathway components and prostate cancer pathogenesis. Gleason score, for example, correlated with the loss of death receptor expression, thus suggesting decreasing apoptosis in the more aggressive and undifferentiated cancers. Furthermore, patients diagnosed at the age of 60 and higher experienced significantly lower levels of death receptors. Interestingly, this correlation might be consistent with the finding that older men experience a higher rate of PSA recurrences,[45] as their tumors become increasingly resistant to TRAIL-induced apoptosis. The concomitant decrease in $FLIP_L$ expression may not influence this phenotype, as diminished death receptor expression already renders the cells sufficiently resistant to TRAIL-induced apoptosis. Any additional $FLIP_L$ downregulation, taking effect downstream of the death receptor, may not offer any benefit reflected in tumor growth. By this reasoning, $FLIP_L$ upregulation leads to decreased apoptosis of the tumor cell—only in case the regular expression of death receptor is unhindered or even increased.

Finally, we propose that the TRAIL pathway might be severely dysregulated in prostate cancer, as virtually all tissue samples on our TMA demonstrated aberrations that eventually lead to TRAIL resistance. Our findings also suggest that most of these aberrations take place at the level of TRAIL receptors or its immediate downstream complex, the DISC, which is inhibited by FLIP. Although, in this study, we cannot rule out additional effects of downstream pathway components on TRAIL sensitivity, nor even investigate the functional impact of these aberrations on apoptosis in vitro or in vivo, the sheer percentage of patients displaying either death receptor downregulation and/or $FLIP_L$ overexpression is startling. Hence, we conclude that TRAIL-based therapy might benefit from an analysis of TRAIL resistance factors before treatment initiation. A therapeutic approach targeting these resistance factors might complement TRAIL and possibly increase its efficacy.

To summarize, we measured protein expression of TRAIL, its receptors DR4, DR5, DcR1, and DcR2, as well as $FLIP_L$ on a prostate cancer tissue microarray in a large cohort. On the basis of correlations with several clinical parameters as well as survival analyses, we suggest that TRAIL pathway plays an important role in prostate cancer pathogenesis and TRAIL expression, in particular, is of high prognostic value. We discovered that stromal expression of TRAIL is lost during transformation from normal prostate to prostate cancer, and this loss is further associated with poor recurrence-free survival of prostate cancer patients.

CONFLICT OF INTEREST DISCLOSURES

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