Expression of TRAIL and TRAIL Death Receptors in Stage III Non-Small Cell Lung Cancer Tumors

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ABSTRACT

Purpose: Several in vitro studies have shown that non-small cell lung cancer (NSCLC) cell lines are sensitive to apoptosis induction by the recombinant human (rh) tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death ligand, indicating that rhTRAIL might become an attractive molecule for treatment of NSCLCs. To investigate the therapeutic potential of rhTRAIL, the expression of TRAIL and its apoptosis-inducing receptors DR4 and DR5 was evaluated in tumors of stage III NSCLC patients.

Experimental Design: Before treatment, tumor biopsies from locally advanced NSCLC patients were obtained by bronchoscopy. DR4, DR5, and TRAIL expression were determined immunohistochemically in 87 tumors. Patients were randomized for treatment with 60 Gy radiotherapy with or without carboplatin as radiosensitizer.

Results: DR4, DR5, and TRAIL were expressed in 99%, 82%, and 91% of the tumors, respectively. Seventeen percent of the samples expressed only DR4 and no DR5. In NSCLCs with squamous cell differentiation, a typical staining pattern for DR4 and DR5 was observed. Cells from the basal layer were strongly positive, and the more mature cells were less positive or negative. An inverse staining pattern was observed for TRAIL. Poorly differentiated areas showed strong staining intensity for DR4, DR5, and TRAIL. DR5-positive staining was associated with increased risk of death (odds ratio, 5.76; 95% confidence interval, 1.04–31.93; P = 0.045).

Conclusions: The majority of the locally irresectable stage III NSCLCs expressed at least one of the two death receptors for TRAIL. Therefore, these death receptors may provide a target for the use of rhTRAIL as a new adjunct in the treatment of stage III NSCLC.

INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality in the Western world. NSCLCs represent 75–80% of all types of lung cancer, and include squamous cell carcinomas, adenocarcinomas, and large-cell carcinomas. For NSCLC patients with early stage disease (stages I and II), surgical resection offers substantial cure rates (1, 2). However, ~70% of the patients present with locally or regionally advanced cancers (stage III) or with metastatic disease (stage IV). Although a combination of chemotherapeutic drugs and radiation therapy has clearly improved treatment results, most of these patients die of disease progression because of acquired or intrinsic resistance to chemotherapeutic drugs (3). Therefore, the investigation of novel agents continues to have a high priority.

Current interest is focused on the so-called “death ligands,” including TNF, FasL, and TRAIL (4–6). Death ligands can trigger apoptosis in tumor cell lines via their cognate cell-surface death receptors, TNFR1, Fas, and DR4/DR5, respectively. In particular, rhTRAIL harbors potential as a cancer therapeutic agent. In preclinical models it has no adverse effects on normal tissues and shows antitumor activity (7–12). In addition, rhTRAIL acts synergistically with chemotherapeutic drugs, and can overcome drug resistance in tumor cell lines and animal tumor models (11–15).

TRAIL can interact with several distinct receptors. Two of these receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2/TRICK2), are plasma membrane proteins containing intracellular death domains essential for the transmission of the death signal on TRAIL binding (16–19). Two other membrane receptors, DcR1 (TRAIL-R3/TRID) and DcR2 (TRAIL-R4/TRUNDD), are so-called decoy receptors because they can bind TRAIL but lack death domains and are unable to induce cell death (18–20). Initially, it was suggested that the relative distribution of DR4 and DR5 versus DcR1 and DcR2 determines the sensitivity of a given cell to rhTRAIL-induced apoptosis (18–20). However, several in vitro studies have failed to find this correlation and suggest that rhTRAIL-induced cell death is regulated by intracellular factors (12, 21–23).

In vitro studies have shown that several NSCLC cell lines are sensitive to apoptosis induction by rhTRAIL, whereas normal lung epithelial cells are resistant (7, 24–29). These studies indicate that rhTRAIL may become an attractive molecule for treatment of NSCLCs. However, no data are available on the DR4 and DR5 protein expression in human NSCLCs. In this...
study, we analyzed immunohistochemically the expression of TRAIL and its apoptosis-inducing receptors, DR4 and DR5, in stage III NSCLC samples.

MATERIALS AND METHODS

Patients and Biopsies. Tumor samples were available from patients who participated in a randomized study (30). Tumor biopsies were obtained before treatment by bronchoscopy. From four patients, tumor samples were not only obtained from the primary tumor but also from the mediastinal lymph nodes. From two patients, material was available only from the mediastinal lymph nodes. All of the NSCLC samples were fixed in 10% formalin and paraffin-embedded. Three-μm sections were cut and placed on 3-aminopropyltriethoxysilan-coated slides. The presence of NSCLC tumor tissue in the sections was confirmed by an independent pathologist using standard H&E staining on parallel sections. Patients with locally inoperable stage III NSCLC were treated with radiotherapy (60 Gy) administered as 2 Gy/day for 5 days a week during 6 weeks with or without continuous i.v. carboplatin (total dose: 860 mg/m² in 6 weeks) as radiosensitizer. The study was approved by the ethics committee. Informed consent was obtained from all of the patients. Tumor response was measured according to the WHO criteria. Patients were followed every 3 months by history, physical examination, chest X-ray, and additional imaging tests when there was reason to suspect metastatic disease. Overall survival was calculated from the time of randomization until death, and censored for loss of follow-up or still alive at the time of closure of this study. Time to progression was calculated from the time of randomization until the time of local tumor progression or the occurrence of metastases.

RT-PCR. High-quality RNA was isolated by lysing 25-μm sections of frozen NSCLC samples in a guanidine thiocyanate buffer [4 M guanidine thiocyanate, 0.5% N-lauroyl sarcosine, 25 mM sodium citrate (pH 7.0), and 0.1 M 2-mercaptoethanol] according to Wisman et al. (31). The quality of the samples was confirmed by agarose gel electrophoresis. Before cDNA synthesis, RNA was treated with DNase I (Roche Diagnostics, Almere, the Netherlands). cDNA was synthesized as described by the manufacturer’s protocol (Life Technologies, Inc., Breda, the Netherlands) using oligodeoxythymidylic acid primers and Moloney murine leukemia virus transcriptase. The PCR programs and primers used to amplify DR4, DR5, and TRAIL were described elsewhere (32). Primer sequences for glyceraldehyde-3-phosphate dehydrogenase were 5'-CAC-CACCATGGAGAAGGCTGG-3' and 5'-CCAAGTGCTGTCA-TGGATGACC-3', which resulted in a 200-bp fragment after 24 cycles. PCR products were electrophoresed in a 2% agarose gel in 1X Tris-borate EDTA buffer. The colon carcinoma cell line SW489 cell line was used as a positive control (33).

Western Blot Analysis. Frozen NSCLC samples were pulverized and dissolved in cold PBS [6 mM NaH2PO4, 1.5 mM KH2PO4, 0.14 mM NaCl, and 2.7 mM KCl (pH 7.2)]. After centrifugation at 23,000 × g for 1 min to remove debris, the supernatant was collected, an equal volume of 2X standard Western blot sample buffer [0.5 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.002% bromphenol blue, and 10% 2-mercaptoethanol] was added, and the samples were boiled for 5 min. Protein concentration was determined according to Bradford (34). All of the samples were size fractionated on SDS-PAGE and transferred onto activated polyvinylidene difluoride membranes (Millipore, Bedford, United Kingdom). After blocking for 1 h in Tris buffer saline supplemented with 5% milk powder (Merck, Darmstadt, Germany) and 0.05% Tween 20 (Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands), immunodetection of DR4, DR5, and TRAIL was performed using the following antibodies: a goat polyclonal IgG specific for DR4 (1:500; clone C-20; Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit polyclonal IgG specific for DR5 (1:500; Oncogene Research, Cambridge, MA), and a goat polyclonal IgG specific for TRAIL (1:100; clone K18; Santa Cruz Biotechnology). Binding of these antibodies was determined using horseradish peroxidase-conjugated secondary antibodies (all from DAKO, Glostrup, Denmark) and visualized with the enhanced chemiluminescence kit of Roche Diagnostics. SW948 cells and soluble rhTRAIL (made according to Ashkenazi et al.; Ref. 7) were used as positive controls.

Immunohistochemistry. Before staining, slides were deparaffinized and dehydrated. For DR5, antigen retrieval was performed by microwave treatment of the slides for 8 min at 700 W in 0.01 M citrate buffer (pH 6.0). For DR4 and TRAIL, no antigen retrieval was performed. Endogenous peroxidase was blocked with 0.3% H2O2 in PBS solution [6.4 mM Na2HPO4·H2O, 1.5 mM KH2PO4, 0.14 mM NaCl, and 2.7 mM KCl (pH 7.8)] for 30 min, followed by incubation with avidin and biotin blocking solutions (Vector Laboratories, Burlingame, CA). For DR4 and DR5, the slides were preincubated with 1% AB serum and 1% BSA (Life Technologies, Inc.) in PBS for 15 min. For TRAIL, 5% normal rabbit serum was used instead of AB serum. The primary antibodies described earlier were applied for 1 h at room temperature. Dilutions of 1:50, 1:100, and 1:25 in PBS 1% BSA were used for DR4, DR5, and TRAIL, respectively. After washing with PBS, slides were incubated with a 1:300 dilution of a biotinylated rabbit-antibody or a swine-antirabbit antibody (DAKO), followed by addition of streptavidin-conjugated peroxidase (DAKO). Peroxidase activity was visualized by incubating the slides for 10 min in 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Chemie BV) solution (0.05% 3,3-diaminobenzidine tetrahydrochloride, 0.1% imidazol, and 0.03% H2O2 in PBS). Counterstaining was performed with hematoxylin for 2 min. As negative controls, the primary antibodies were substituted with nonimmune normal goat or normal rabbit IgGs. For DR4 and TRAIL, immunohistochemistry was also performed in the presence or absence of a 10-fold excess of a corresponding blocking peptide (Santa Cruz Biotechnology). Positive controls were (tumor) tissue samples, found on previous occasions to stain positive: normal liver tissue, first trimester placenta tissue, colon, or ovarian cancer tissues. Control archival normal lung samples (n = 4) were obtained from noninvolved parts of lobectomy specimens, removed in therapeutic surgical procedures for bronchial carcinoma.

Staining Analysis. All of the sections were reviewed light microscopically by three observers (D. C. J. S., S. d. J., W. T.), without knowledge of the clinical data. Tumors showing ≤10% positively stained cells were considered negative. Samples showing >10% positively stained tumor cells were consid-
erated positive. All of the stained tumor biopsies were evaluated semiquantitatively for intensity of staining: no staining (0), weakly positive staining (1), positive staining (2), or strong positive staining (3). The percentage of positive cells, localization of staining (nuclear, membranous, or cytoplasmic), heterogeneity, and pattern of staining regarding differentiation grade were also recorded. For statistical analysis, all of the tumors with positive or strong positive staining in >10% of tumor cells were considered positive, whereas tumors with negative or weakly positive staining were considered negative.

Statistics. Data analysis was performed using SPSS 10.0 software package (SPSS Inc., Chicago, IL). Associations among DR4, DR5, and TRAIL staining, and the relationship among DR4, DR5, and TRAIL staining and tumor response were evaluated by the χ² test or Fisher’s exact test when appropriate. Kaplan-Meier curves were used to compare overall and progression-free survival rates between groups. Statistical differences in survival between tumors staining positive or negative for DR4, DR5, or TRAIL were analyzed using the log-rank test. Multiple logistic regression models were used to estimate the risk of death for positive compared with negative DR4, DR5, or TRAIL staining. All three of the immunohistochemical factors were adjusted for each other and histopathology, stage, lymph nodes (N1, N2, and N3), sex, and duration of follow-up. The risk of recurrence according to DR4, DR5, and TRAIL staining were estimated likewise. All of the tests were performed two-sided, and Ps < 0.05 were considered statistically significant.

RESULTS

Patient Characteristics and Tumor Biopsies. NSCLC tumor samples were used as described in a study by Fokkema et al. (35). Briefly, bronchoscopic evaluation was performed in 155 of 160 patients who entered a randomized study assessing the radiosensitizing effect of carboplatin in stage III NSCLC (30, 35). Biopsies were taken from 117 patients with central bronchial tumors for histological diagnosis, and from 95 of these patients residual tumor was available for additional studies. The characteristics of these patients are shown in Table 1. The patients were equally distributed between treatment with radiotherapy alone and treatment with radiotherapy combined with carboplatin (Table 1). Expression of DR4, DR5, and TRAIL was equally distributed over both treatment arms. The addition of carboplatin had no effect on response or survival (30), so no distinction was made between the treatment arms in additional analysis. Paraffin-embedded tissue was available from 95 patients, and 74–87 of these had enough remaining evaluable tissue for subsequent immunohistochemical staining.

Detection of DR4, DR5, and TRAIL mRNA and Protein. To validate the specificity of the antibodies used in the immunohistochemical staining, additional analyses were performed on five resectable NSCLC samples. First, the DR4, DR5, and TRAIL mRNA expression was evaluated by RT-PCR. Fig. 1A shows that all five of the NSCLCs expressed mRNA for both death receptors and TRAIL. Second, the protein expression was checked in these five NSCLCs by immunoblot analysis using the same antibodies for DR4, DR5, or TRAIL detection as used for immunohistochemical staining. The SW948 cell line served as a positive control for DR4 and DR5, and soluble rhTRAIL for the TRAIL staining. The TRAIL antibody used in this study is directed against the extracellular COOH terminus of the TRAIL protein and should, therefore, recognize both soluble and membrane-bound TRAIL. As shown in Fig. 1B, the antibodies detected protein products corresponding to the predicted molecular mass: ~50 kDa for DR4 and DR5 in SW948 cell line and 20 kDa for soluble rhTRAIL (7, 8). All five of the NSCLC expressed DR4 protein, although more bands were detected that were slightly higher than in SW948 cells. Because DR4 has an N-linked glycosylation site (16), these bands could represent different glycosylated forms of the DR4 protein. DR5 protein was detectable in all of the NSCLC samples at approximately 46–50 kDa as described by Walczak et al. (17). The antibody used for TRAIL protein detection showed several bands at ~32, 40, and 60 kDa corresponding to membrane-bound TRAIL, and dimer and trimer forms of (soluble) TRAIL (7, 8). Some lower bands seen in P3 and P4 could possibly correspond to a truncated or degraded TRAIL fragment. TRAIL protein was found in all of the tumor samples. From two of these NSCLC samples, P1 and P3, immunohistochemical stainings of DR4, DR5, and TRAIL are shown in Fig. 1, C–H. The immunohistochemical and immunoblot stainings were in accordance, indicating the specificity of the antibodies used for DR4, DR5, and TRAIL detection. Preincubation of the DR4 or TRAIL antibodies with the corresponding blocking peptide completely abolished the immunostaining, additionally confirming the specificity of the antibodies. Moreover, no staining was observed when the primary antibodies were replaced by nonimmune normal goat (DR4 and TRAIL) or rabbit (DR5) IgGs.

Expression of DR4, DR5, and TRAIL Protein in Stage III NSCLC. Both DR4 and DR5 staining was cytoplasmic, and no clear membranous staining was detected. TRAIL was expressed mainly in the cytoplasm but sometimes nuclear staining was also observed. The intensity of DR4, DR5, and TRAIL expression differed considerably between the tumors, as did the expression pattern (diffuse versus granular staining). Both DR4 and DR5 were detected in bronchial epithelial cells of all of the histologically normal control lung tissues studied (Fig. 2, A and B). However, in contrast with the overall cytoplasmic staining in tumor cells, in normal bronchial epithelium expression of the receptors was located at the apical side of the bronchial epithelial cells with a similar intensity as in tumor cells. The bronchial epithelium was negative or only weakly positive for TRAIL in two of four normal lungs and moderately positive in the other two.

Table 2 shows the staining characteristics of DR4, DR5, and TRAIL in the stage III NSCLC samples tested. Examples of
DR4, DR5, and TRAIL expression in NSCLCs are shown in Fig. 2, C–H. DR4 was detectable in all but one of the tumor samples studied and was strongly expressed in 70% of the NSCLCs. DR5 expression was generally lower than DR4 expression and was not detectable in 18% of the tumors studied. Twenty percent showed strong DR5 expression. Only one NSCLC expressed neither DR4 nor DR5. Seventeen percent of the studied samples expressed only DR4 and no DR5. Lack of TRAIL expression was found in 9% of the NSCLC samples. Strong TRAIL expression was found in 59% of the studied NSCLCs. DR4, DR5, and TRAIL were heterogeneously expressed in 81, 78, and 82%, respectively, of the positively stained NSCLCs. Interestingly, NSCLCs with squamous cell differentiation often showed a typical DR4 staining pattern in which cells in the basal layer were strongly positive, and the more mature cells in the supra basal layers were less positive or even negative for DR4 (Fig. 2D). For DR5, the same staining pattern was observed as for DR4, although this was sometimes less obvious (Fig. 2F). In contrast, an inverse staining pattern was observed for TRAIL with the basal cells being less positive compared with the mature cells (Fig. 2H). Poorly differentiated areas within the well-differentiated NSCLC tumor specimens showing no clear distinction between a basal and supra basal cell layer often revealed strong staining for DR4, DR5, and TRAIL.

DR4 and DR5 expression were not associated \((n = 87; P = 0.124)\). In contrast, there was a significant association between DR4 and TRAIL expression \((n = 74; P = 0.020)\), and between DR5 and TRAIL expression \((n = 74; P = 0.034)\). Considering the different tumor types, there were more large-cell carcinomas

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**Fig. 1** mRNA and protein expression of DR4, DR5, and TRAIL in NSCLC tumors. DR4, DR5, and TRAIL mRNA, and protein expression of five resectable NSCLC tumors \((P1–P5)\) were determined by RT-PCR \((A)\) and Western blot analysis \((B)\), respectively. As positive control, \(C\), served SW948 or soluble rhTRAIL. Immunohistochemical staining was performed for DR4 \((C \text{ and } D)\), DR5 \((E \text{ and } F)\), and TRAIL \((G \text{ and } H)\) on two NSCLC samples, \(P1 (C, E, \text{ and } G)\) and \(P3 (D, F, \text{ and } H)\).
(4 of 9; 44%) with low or no TRAIL expression compared with squamous cell carcinomas (5 of 47; 11%) or adenocarcinomas (5 of 18; 18%; \( P = 0.033 \)). No significant correlation was found between the DR4 or DR5 expression and the different NSCLC tumor types.

**Relation of Expression of DR4, DR5, or TRAIL on Tumor Response and Prognosis.** Tumor response was not associated with DR4, DR5, or TRAIL expression. Kaplan-Meier survival curves indicate (initial) better survival for negative DR4, DR5, or TRAIL staining compared with positive staining (Fig. 3, A, C, and E, respectively). These differences were not statistically significant. Likewise, DR4, DR5, and TRAIL staining were not associated significantly with time to progression as outcome variable (Fig. 3, B, D, and F, respectively). Because only four NSCLC samples revealed no or only weakly positive DR4 staining, DR4 expression was additionally excluded from the statistical analyses. To estimate the risk of death according to DR5 and TRAIL independent of each other and confounding factors [histopathology, stage, lymph nodes (N1, N2, and N3), sex, and duration of follow-up], we performed multiple logistic regression. DR5-positive staining was associated significantly with an increased risk of death (OR, 5.76; 95% CI, 1.04–31.93; \( P = 0.045 \)). TRAIL-positive staining was not associated with increased risk of death (OR, 1.72; 95% CI, 0.33–8.90; \( P = 0.34\)).
Table 2 Immunohistochemical staining results in stage III NSCLC patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>DR4</th>
<th>DR5</th>
<th>TRAIL</th>
</tr>
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<tr>
<td>No. patients*</td>
<td>87</td>
<td>87</td>
<td>74</td>
</tr>
<tr>
<td>No. positive staining</td>
<td>86</td>
<td>71</td>
<td>67</td>
</tr>
<tr>
<td>Staining intensity</td>
<td></td>
<td></td>
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<tr>
<td>0</td>
<td>0</td>
<td>1</td>
<td>7</td>
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<tr>
<td>1</td>
<td>3</td>
<td>32</td>
<td>7</td>
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<tr>
<td>2</td>
<td>22</td>
<td>21</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>61</td>
<td>18</td>
<td>44</td>
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<tr>
<td>Histology</td>
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<td></td>
<td></td>
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<tr>
<td>Squamous cell</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>No. positive staining</td>
<td>53</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>No. negative staining</td>
<td>0</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>No. positive staining</td>
<td>22</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>No. negative staining</td>
<td>1</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Large cell</td>
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<td></td>
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<tr>
<td>No. positive staining</td>
<td>11</td>
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<td>2</td>
<td>1</td>
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</table>

*Patients with biopsies that contained sufficient detectable tumor cells to make a proper evaluation of the immunohistochemical staining. Biopsies containing too much necrosis, background, or absence of proper tissue that could be defined were considered nonevaluable.

Whether the observed DR4 and DR5 expression in NSCLCs can be translated into a successful rhTRAIL therapy also depends on the functionality of these receptors. Two missense mutations in the DR4 gene have been detected in NSCLCs (39). These alterations result in amino acid changes in or near DR4' ligand-binding domain, which might interrupt TRAIL binding to DR4. However, this assumption is based only on the crystal structure of DR5 and its homology with DR4, and still has to be proven. DR5 gene mutations in the death domain, known to be involved in the transduction of the apoptotic signal, were observed in 10.6% of NSCLCs in a Korean population (40). However, in white Americans, no DR5 mutations were found in 100 NSCLCs (41). The same study showed DR5 mRNA expression in 30 primary NSCLCs. In our study, DR5 protein expression was absent in 18% of the NSCLCs.

DISCUSSION

In the present study, the expression of the apoptosis-inducing TRAIL receptors, DR4 and DR5, was studied to investigate the therapeutic potential of rhTRAIL for NSCLC. All but one of the NSCLCs showed protein expression of at least one of the two death receptors for TRAIL. DR4 was present in almost all of the tumors (99%), whereas DR5 was expressed in 82%.

The presence of these death receptors in NSCLCs is interesting for future treatment of stage III NSCLC patients with rhTRAIL. The success of rhTRAIL therapy will, among others, be dependent on the presence of the death receptors on the cell surface (22, 23). For example, rhTRAIL-sensitive Ewing’s sarcoma cell lines expressed significant levels of DR4 and DR5 on their cell surface, whereas a resistant cell line lacked expression of these death receptors (22). Restoration of the surface DR5 levels rendered the rhTRAIL-resistant cell line sensitive to rhTRAIL. In the present study, DR4 and DR5 expression was determined immunohistochemically in paraffin-embedded sections. Both receptors were detectable, but no membranous staining pattern was observed. However, this does not preclude the presence of DR4 or DR5 on the cell surface, because immunohistochemistry is not an optimal technique to detect membranous staining. Moreover, Zhang et al. (36) demonstrated that, although present on the cell surface, both DR4 and DR5 are predominantly located in the trans-Golgi network. Furthermore, they suggested that the localization in the trans-Golgi network might be a common feature of apoptosis-inducing TNF family receptors, because Fas and TNF were also reported to be located in the trans-Golgi network (37, 38).

Chemotherapy and ionizing radiation can render tumor cells more sensitive to rhTRAIL-induced apoptosis (11–15). In addition, combination of rhTRAIL and chemotherapeutic drugs can even overcome drug resistance. Up-regulation of DR4 or DR5 levels and increased membrane localization are important mechanisms, which could explain an increased sensitivity to rhTRAIL. However, in some models abolition of rhTRAIL resistance by chemotherapeutic drug was caused by intracellular mechanisms and not by alteration of death receptor levels. Several inhibitors of the TRAIL signaling pathway have been described, such as FLICE-like inhibitory protein, inhibitor of apoptosis proteins, or antiapoptosis members of the Bcl-2 family like Bcl-2/Bcl-XL (44). However, none of these factors correlated with the degree of sensitivity to rhTRAIL or the level of apoptosis after chemotherapy in NSCLC cell lines (26, 47, 48). In vitro studies demonstrated that NSCLC cell lines could be sensitized to rhTRAIL induced apoptosis by cisplatin, camptothechin, paclitaxel or the synthetic retinoid 6-[3-(1-Adaman-tyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437: 24–26). Therefore, chemotherapy combined with rhTRAIL could potentially render NSCLCs more susceptible to rhTRAIL-induced apoptosis.

A number of studies have demonstrated that patients without expression of death receptor Fas in their NSCLC had a shorter survival than patients with Fas-positive NSCLC (49, 50). This may indicate that Fas is an important regulator of lung cancer growth and increases the sensitivity of the tumor to physiological apoptotic signals induced by FasL from the tumor self or the immune system. A few studies have demonstrated recently a physiological role for TRAIL in tumor surveillance by natural killer cells in mice and human NSCLC tumor-infiltrating lymphocytes in SCID mice (29, 51, 52). Until now, no data were available about the effect of the TRAIL receptors DR4 and DR5 on the clinical outcome of NSCLC patients. In our study, DR5 expression was associated with an increased risk.
of death. This indicates that DR5-positive NSCLCs may have a growth advantage. Interestingly, recent reports demonstrated that besides apoptotic signals, a number of other signaling pathways are activated by death receptors such as the extracellular-signal regulated kinase pathway (53–55). Activation of extracellular-signal regulated kinase 1/2 by the death ligands FasL, TRAIL, and TNF seems to have a dominant protecting effect over the apoptotic signal from their death receptors resulting in proliferation rather than apoptosis. Therefore, the death receptor/ligand systems may have an important role in tumorigenesis by providing an autocrine growth factor and, thus, promoting tumor growth.

TRAIL was expressed by 91% of the NSCLCs. It was suggested that the expression of apoptosis-inducing ligands such as TRAIL may play an important role in cell regulation and may result in an immunological advantage for tumor cells (56, 57). Although the Kaplan-Meier survival curve indicates an initial longer survival for TRAIL-negative NSCLCs compared with TRAIL-positive tumors, no significant association was observed between loss of TRAIL expression and overall survival. Interestingly, however, we noticed that NSCLCs with squamous cell differentiation often showed a typical staining pattern for DR4 or DR5 with the basal cells being highly positive and the more mature cells in the supra basal layers less positive or negative. An inverse staining pattern was observed for TRAIL. Poorly differentiated areas next to well-differentiated areas often showed strong staining intensity for DR4, DR5, and TRAIL.

In conclusion, in the present study we have shown expression of DR4 and DR5 in locally irresectable stage III NSCLCs. Systemic administration of rhTRAIL has already appeared to be...

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**Fig. 3** Cumulative survival in patients with stage III NSCLC. Kaplan-Meier estimates of overall survival (A, C, and E) and progression-free survival (B, D, and F) according to DR4 (A and B), DR5 (C and D), and to TRAIL (E and F) expression. All tumors with positive or strong positive staining in >10% of tumor cells were considered positive, whereas tumors with negative or weakly positive staining were considered negative.

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remarkably safe and nonimmunogenic in mice and nonhuman primates (7–9). Moreover, Lawrence et al. (8) showed that, in contrast with polyhistidine- and cross-linked flag-tagged versions, the native sequence rhTRAIL is not toxic to human and cynomolgus monkey hepatocytes. These observations suggest a potential value of rhTRAIL for cancer therapy. Although NSCLC is relatively resistant to chemotherapy and radiation, the expression of both TRAIL death receptors may provide a basis for the use of rhTRAIL in combination with chemotherapy as a new approach to treat stage III NSCLC.

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