Role and Prognostic Significance of Tumor Necrosis Factor-related Apoptosis-inducing Ligand Death Receptor DR5 in Nonsmall-Cell Lung Cancer and Precursor Lesions

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BACKGROUND. The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor, DR5, mediates proapoptotic signals and is implicated in the pathogenesis of many neoplasms including nonsmall-cell lung cancer (NSCLC).

METHODS. In this study, immunohistochemical expression of DR5 was examined in 146 cases of stage I and II NSCLC as well as neoplastic precursor lesions and regional lymph node metastases using tissue microarrays.

RESULTS. High DR5 expression was observed in 67.1% of primary NSCLC, 55.6% of bronchial squamous carcinoma in situ, 40% of squamous metaplasia, as well as 76.5% of lymph node metastases. In all of these lesions, DR5 expression was significantly higher than in normal bronchial epithelium. Increased expression of DR5 correlated with poorly differentiated tumors and was inversely correlated with bronchioloalveolar carcinomas. There was no correlation with other clinicopathologic variables. A significant association was found between high DR5 expression and reduced overall survival in univariate analysis. Among smokers, high DR5 and tumor stage were independent predictors of reduced disease-free survival in multivariate analysis, however, DR5 was not an independent prognostic marker among the entire cohort of NSCLC.

CONCLUSIONS. These findings suggest that DR5 plays a role in the development of early-stage NSCLC and the high levels of DR5 expression suggest that these tumors may be susceptible to novel anticancer agents targeting the DR5 receptor and may improve patient survival, particularly for patients who are smokers. ***Cancer* 2008;113:135–42.** © *2008 American Cancer Society.*

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Although apoptosis plays an important role in eliminating damaged cells, abnormalities in the regulation of apoptosis can cause an imbalance between proliferation and cell death and contribute to tumorigenesis and drug resistance.[1] The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family that can bind to 4 membrane-bound receptors

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involved in the transmission of apoptotic regulatory signals via the extrinsic pathway.[2] Two of the TRAIL receptors, death receptor 4 (DR4/TRAIL-R1) and death receptor 5 (DR5/TRAIL-R2), are agonistic and mediate apoptosis via a homologous intracellular death domain. The other 2 TRAIL receptors (R3, R4) are antagonistic 'decoy' receptors that compete for TRAIL binding but cannot transmit an apoptotic signal as they lack functional intracellular death domains.

Interest in the TRAIL pathway has arisen as its proapoptotic characteristics could potentially be exploited in the treatment of cancer. TRAIL has demonstrated selective killing of tumor cells in animal models and in several tumor cell lines including lung cancer.[3–5] Moreover, in preclinical models TRAIL has shown no significant adverse effects on normal tissues unlike other tested members of the TNF family.[3,6] Agonistic monoclonal antibodies directed against DR4 and DR5 have demonstrated selective antitumor activity in vitro and in mouse xenograft models of multiple tumor types including NSCLC[7] and phase 2 clinical trials are currently being undertaken.[8]

Lung cancer is 1 of the most common human malignancies and is a leading cause of cancer-related deaths in the Western world.[9] The prognosis of early-stage NSCLC is poor with overall 5-year survival rates of about 65% in stage I disease and 40% in stage II.[10] Many patients develop relapses at extrathoracic sites despite receiving the standard treatment of surgical resection and adjuvant chemotherapy.[9] Greater understanding of the molecular mechanisms underlying lung carcinogenesis and improved prognostic information could assist in the management of NSCLC. Targeted molecular anticancer agents such as those acting on the TRAIL pathway may be of additional benefit in some patients.

Previous studies have implicated deregulation of the TRAIL pathway as a means of evading apoptosis in many human tumors including NSCLC. Although the prognostic significance of TRAIL death receptors in late-stage NSCLC has been investigated in 2 studies,[11,12] little is known of their role in early-stage NSCLC. We investigated the expression of the proapoptotic death receptor DR5 in primary early-stage NSCLC, precursor lesions, and regional metastases by examining protein expression and correlating the results with clinicopathologic variables and patient survival.

MATERIALS AND METHODS

Patient Cohort

Tumor samples and clinical follow-up data were obtained from a cohort of 146 stage I–II NSCLC patients treated at the Royal Prince Alfred Hospital, Sydney, Australia, between 1996 and 1999. The cohort included 90 (61.6%) men and 56 (38.4%) women, with a median age at diagnosis of 68 years (range, 41–87 years) and median survival time of 64.5 months (range, 1–103 months). Smoking history was available for 143 patients, of which 135 (94.4%) were current or ex-smokers and 8 (5.6%) were classified as nonsmokers. None of the patients received adjuvant chemotherapy. Histologic tumor subtypes were assessed using the WHO classification[13] and there were 69 (47.3%) adenocarcinomas (ADCs) (including 10 bronchioloalveolar carcinomas [BACs]), 16 (11.0%) large cell carcinomas (LCCs), and 61 (41.8%) squamous cell carcinomas (SCCs). Tumors were staged using the AJCC TNM classification[14] and consisted of 118 (80.8%) stage I and 28 (19.2%) stage II tumors. The cohort included 17 patients with regional lymph node metastases. Precursor lesions were also assessed when available and there were 10 cases of bronchial squamous epithelial metaplasia, 3 with low-grade dysplasia, and 9 cases of bronchial SCC in situ. Follow-up information of at least 5 years was available for this study.

Tumor Samples

Tissue microarrays were constructed using 3 to 4 donor cores of tumor, 1 mm in diameter, from appropriate areas in formalin-fixed, paraffin-embedded tissue blocks as previously described.[15] These tissue cores were arrayed in a recipient paraffin block using a tissue arraying instrument (Beecher Instruments, Silver Springs, Md). Serial sections were cut from the tissue microarray blocks at 4 μm thickness and mounted on glass slides.

Immunohistochemical Staining

After deparaffinization in xylene and rehydration of the tissue sections through graded decreasing concentrations of alcohol, antigen retrieval was performed by adding EDTA, pH 8.6, buffer, and by heating in a microwave 3 times for 5 minutes each time. Primary mouse antihuman monoclonal antibody against DR5 (courtesy of Dr. Gavin Screaton) was applied to the sections according to previously described methods.[16,17] The sections were incubated at room temperature for 1 hour at 1:200 dilution in Tris buffer. The Vectastain ABC kit (Vector Laboratories, Burlingame, Calif) was used to detect the monoclonal antibody according to the manufacturer's specifications, and the binding sites were observed using the DAB kit (Dako, Carpinteria, Calif). The sections were counterstained with Harris hematoxylin. Internal controls of matched samples of normal

bronchial mucosa and peripheral lung parenchyma were incorporated into the tissue arrays. Samples from normal spleen were also used in the arrays as both reference points and as external controls (positive staining within marginal zone lymphocytes but not within follicle centers). Negative controls were also performed by omission of the primary antibody. Sections of colonic adenocarcinoma and positively staining melanoma were used as positive controls.[17]

Scoring

Two pathologists (W.C. and S.L.) independently scored each case without knowledge of the patient's clinical details and an average of the 2 scores was used. Immunohistochemical expression of DR5 was scored semiquantitatively by estimating the percentage of cells with positive staining. Cellular localization of staining was also assessed. An average score was obtained from the multiple samples of each case and high protein expression was taken as cases with 100% of cells staining positively. Where markedly discrepant, the case was reviewed before deciding on an appropriate consensus score. There was good correlation between the scores obtained from each pathologist (correlation coefficient R = 0.89, $P < .001$).

Statistical Analyses

The chi-square test was used to compare the DR5 expression in primary carcinoma and other lesions. Associations between protein expression and various clinicopathologic characteristics were compared using the Pearson chi-square test and Fisher exact test (2-sided). The Kaplan-Meier log-rank and Cox proportional regression model were used for survival analyses. SPSS statistical software package v. 13.0 (Chicago, Ill) was used for all analyses. *P*-values of $<.05$ were regarded as statistically significant.

RESULTS

DR5 Protein Expression in Normal Lung

Staining for DR5 was seen in most samples of normal bronchial epithelium and lung alveolar epithelium (Fig. 1). In bronchial epithelium there was expression of DR5 along the ciliated apical surface as well as weak cytoplasmic staining in an average of 46.4% ± 34.6% of cells (mean ± SD) with a median 45.8%. In some cases, weak nuclear staining was also seen in a few basal nuclei. Weak cytoplasmic staining was also observed in bronchial smooth muscle cells, some stromal fibroblasts, and endothelial cells.

DR5 Expression in Primary NSCLC

Expression of DR5 in NSCLC was demonstrated immunohistochemically by positive cytoplasmic staining (Fig. 1). Membranous accentuation of staining was identified in a few cases, particularly those with mucinous or clear cytoplasm. Weak nuclear staining was seen in very occasional cases only. High expression of DR5 was observed in the majority of tumor samples and the percentage of positively staining cells ranged from 0% to 100%. The mean value of tumor cells expressing DR5 was 90.90% ± 17.85% (mean ± SD) and the median was 100%. High expression of DR5 was seen in 67.1% (98 of 146) of cases (using the median score as a cutoff). DR5 expression in primary carcinomas was significantly higher than in normal bronchial epithelium ($P < .001$, chi-square test). In SCC, DR5 expression was high in 31 of 61 cases (50.8%), in LCC 12 of 16 (75.0%), and in adenocarcinomas 35 of 69 (50.7%).

DR5 Expression in Neoplastic Precursor Lesions

Of 9 separate cases of squamous carcinoma in situ found within bronchial epithelium, the mean percentage of cells expressing DR5 was 79.3% ± 27.9% and the median was 100%. Five cases (55.6%) showed high DR5 expression. Ten cases of bronchial epithelial squamous metaplasia without dysplasia were available for assessment. Four (40%) showed a high DR5 expression and the mean was 74% ± 30.7%. There were only 3 cases of low-grade squamous dysplasia within metaplastic bronchial epithelium and none had a high DR5 expression (mean 77%).

DR5 expression in the cases of both carcinoma in situ (CIS) and squamous metaplasia were significantly higher than in normal bronchial mucosa ($P < .001$ and $P = .018$, respectively, chi-square). There was no significant difference when DR5 expression in CIS and squamous metaplasia was compared with all primary NSCLC ($P = .88$ and $P = .41$, respectively) or all primary SCCs ($P = .79$ and $P = .49$, respectively).

DR5 Expression in Regional Lymph Node Metastases

There were 17 separate patients with lymph node metastases available for assessment. Nine of the cases were SCCs, 4 were adenocarcinomas, and 4 were LCCs. In these samples of metastases the mean DR5 expression was 91.6% ± 21.1% and the median was 100%. A high frequency of DR5 expression was seen in 13 of 17 (76.5%) of cases. DR5 expression in regional lymph node metastases was significantly higher than in normal bronchial epithelium ($P < .001$). There was a trend for lymph node metastases to

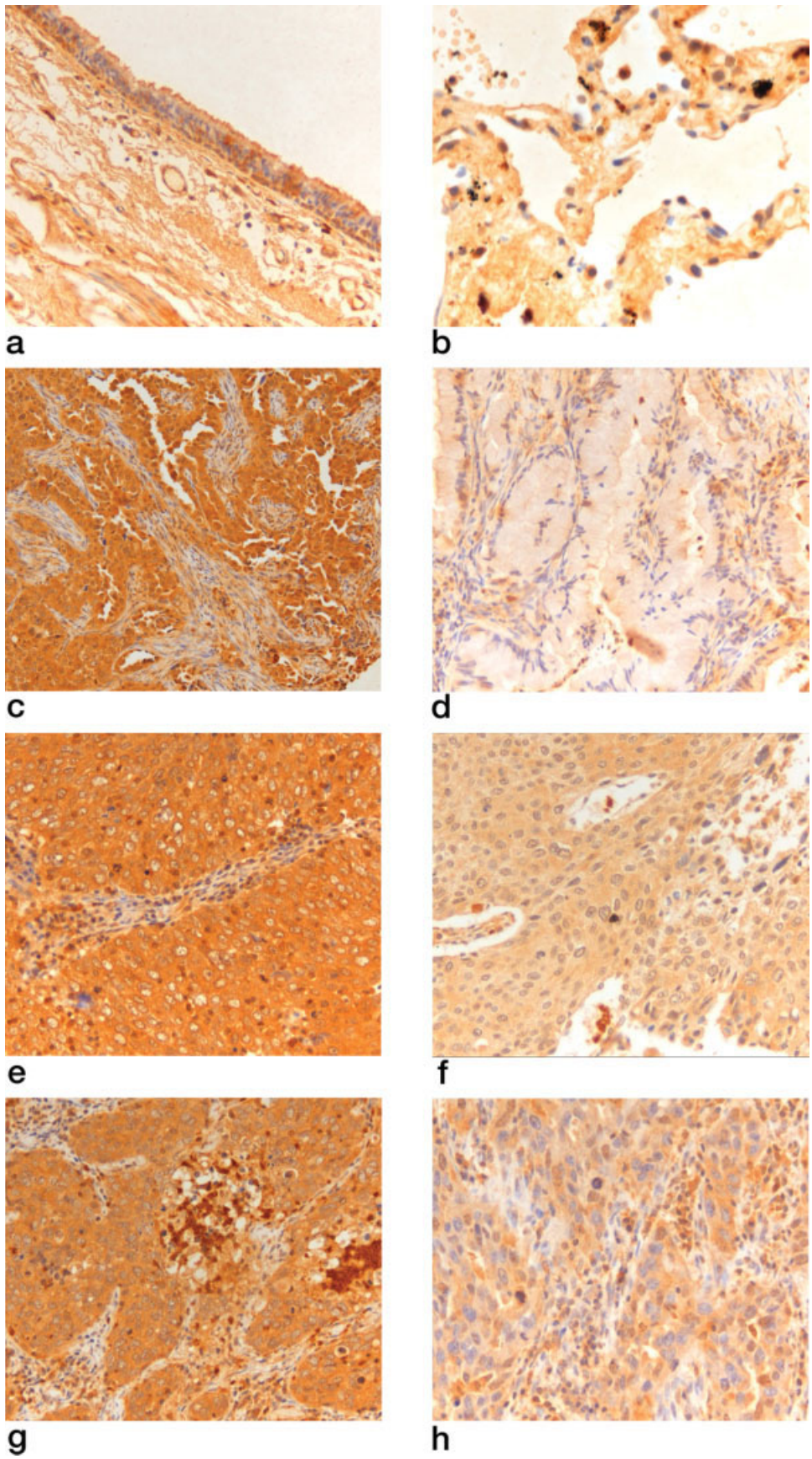

FIGURE 1. Immunohistochemical demonstration of death receptor 5 (DR5) in (a) normal bronchus, (b) normal peripheral lung parenchyma, (c) adenocarcinoma with high DR5 expression, (d) adenocarcinoma (BAC) with low DR5 expression, (e) squamous cell carcinoma (SCC) with high DR5 expression, (f) SCC with low DR5, (g) large cell carcinoma (LCC) with high DR5 expression, (h) LCC with low DR5.

TABLE 1
Relation Between DR5 Expression and Clinicopathologic Characteristics of Patients

	No. high DR5	No. low DR5	Chi-square *P*	Fisher exact test *P*
Tumor type			.068	
ADC	33	26	.62	.74
BAC	2	8	.028*	.045*
SCC	31	30	.59	.62
LCC	12	4	.067	.11
Differentiation			.02*	
Well	4	8	.15	.23
Mod	38	43	.078	.096
Poor	36	17	.008*	.010*
Size			.19	.24
≤30	40	27		
>30	37	390		
Sex			.71	.74
Male	47	43		
Female	31	25		
Age			.83	.87
<67	33	30		
≥67	45	38		
Smoking status			.36	.47
Nonsmoker	3	5		
Smoker	73	62		
Stage			.31	
1A	21	12	.18	.23
1B	40	45	.69	.092
2A	2	2	.89	1.00
2B	15	9	.33	.38
BVI			.84	1.00
Absent	74	65		
Present	4	3		
LVI			.41	.50
Absent	72	65		
Present	6	3		
PNI			.76	1.00
Absent	75	66		
Present	3	2		
Margin			.075	.088
Not involved	68	65		
Involved	10	3		

ADC indicates invasive adenocarcinoma only; BAC, bronchioloalveolar carcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma; BVI, blood vessel invasion; LVI, lymphatic vessel invasion; PNI, perineural invasion.
* Statistically significant at $P < .05$.

show higher expression of DR5 than primary carcinomas but this was not statistically significant ($P = .053$).

Correlation With Pathological and Clinical Variables

The Pearson chi-square test was used to compare the frequency of DR5 alterations with various clinicopathologic characteristics (Table 1). High expression of DR5 was correlated with tumor differentiation ($P = .02$, Pearson chi-square) and poorly differentiated carcinomas ($P = .008$, Pearson chi-square; $P = .01$, Fisher exact test). DR5 expression showed an inverse correlation with bronchioloalveolar carcinomas ($P = .028$, Pearson chi-square; $P = .045$, Fisher exact test 2-sided). There was no correlation with other histologic subtypes, tumor size, tumor stage, lymphovascular invasion, perineural invasion, bronchial margin involvement, patient sex, age, or smoking status.

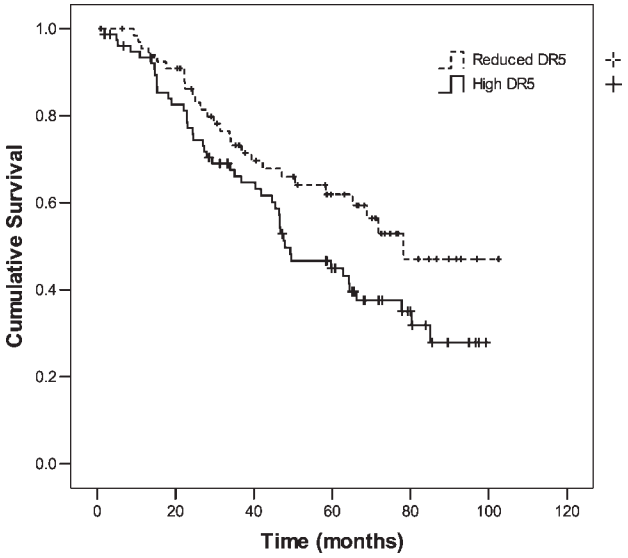

FIGURE 2. Probability of survival according to proportion of cells expressing death receptor 5 (DR5). Kaplan-Meier survival curve, $P = .045$, log rank. Low DR5 expression n = 68 (dotted line), high DR5 expression n = 78 (solid line). There were 73 censored cases.

DR5 Protein Expression and Patient Survival

Expression of DR5 was compared with overall patient survival using Kaplan-Meier survival analysis. High DR5 expression correlated significantly with a worse overall survival ($P = .045$, log rank) (Fig. 2) but was only of borderline significance in predicting disease-free survival ($P = .054$, log rank). Similarly, using univariate Cox regression analysis there was a significant correlation between the proportion of cells expressing DR5 and poor overall survival ($P = .047$, hazard ratio 1.62, 95% confidence interval [CI], 1.007–2.607) but not disease-free survival ($P = .057$, hazard ratio 1.57, 95% CI, 0.99–2.50). Univariate survival analysis was also undertaken on the different clinical and pathologic subsets in the cohort. Analyses based on tumor subtypes, differentiation, stage, and patient sex did not show any significant association between DR5 and survival. Among smokers, high DR5 was predictive of a shorter disease-free survival ($P = .023$, log rank) and overall survival ($P = .031$).

TABLE 2
Results of Cox Regression Analysis Among All NSCLC Patients (N = 146)

	Disease-free survival			Overall survival		
Covariate	*P*	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI
Stage (2 vs 1)	.023	1.82	1.09–3.04	.012	1.94	1.15–3.27
Age	.046	1.03	1.00–1.06	.022	1.04	1.01–1.07
Vessel invasion	.058	1.86	0.98–3.55	.028	2.06	1.08–3.94
DR5 expression	.168	1.40	0.87–2.26	.150	1.43	0.88–2.32

TABLE 3
Results of Cox Regression Analysis Among NSCLC Patients Who Were Smokers (N = 135)

	Disease-free survival			Overall survival		
Covariate	*P*	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI
Stage (2 vs 1)	.035	1.79	1.04–3.08	.012	2.02	1.17–3.50
Age	.124	1.02	0.99–1.06	.020	1.04	1.01–1.07
Vessel invasion	.098	1.74	0.90–3.36	.028	2.08	1.08–3.99
DR5 expression	.029	1.75	1.06–2.88	.122	1.50	0.90–2.52

To examine the importance of DR5 as a predictor of prognosis relative to the effects of different covariates, Cox regression analysis was undertaken. Initially, univariate analysis was undertaken on clinicopathologic variables to determine whether they affected overall survival. The only significant clinical variable predictive of overall survival among all NSCLC patients was stage ($P = .04$), whereas age ($P = .05$) and vascular invasion ($P = .05$) showed borderline significance. These variables along with DR5 expression were used in the Cox regression model using a stepwise forward method. In multivariate analysis, high stage (2 vs 1) ($P = .012$), increasing age ($P = .022$), and the presence of vessel invasion (lymphatic or blood vessel) ($P = .028$) were all independent risk factors for reduced survival (Table 2). Despite showing statistical significance in univariate analysis, the percentage of cells expressing DR5 among all NSCLC patients was not an independent prognostic factor in multivariate analysis based on overall survival ($P = .15$) or disease-free survival ($P = .17$). Similarly, DR5 was not an independent predictor of survival when cases of stage I or stage II disease were analyzed alone (data not shown). Among patients who were smokers, however, DR5 was an independent predictor of shorter disease-free survival ($P = .029$) but not overall survival ($P = .122$) (Table 3).

DISCUSSION

We have demonstrated increased expression of DR5 in NSCLC and neoplastic precursor lesions compared with normal bronchial epithelium, suggesting deregulation of DR5 plays a role in the early stages of NSCLC carcinogenesis. Cytoplasmic DR5 expression was seen diffusely in the majority of primary NSCLC cases and persists in neoplastic progression to regional lymph node metastases, suggesting NSCLC is likely to be susceptible to anticancer drugs targeting the receptor. High expression of DR5 in primary early-stage NSCLC was an adverse prognostic marker in univariate but not multivariate analysis based on all patients in the cohort. Among smokers, high DR5 was an independent marker of shorter disease-free survival, suggesting proapoptotic signals typically associated with DR5 do not have a predominant effect in this setting.

Concordant with the findings in our study, others have demonstrated higher expression of DR5 in lung cancer compared with normal lung tissue.[16] DR5 and DR4 are expressed in a range of other tumors at higher levels than their normal counterparts, including colon,[18] breast,[19] cervix,[20] pancreas[21], and melanoma.[17] TRAIL induces selective killing of tumor cells in vitro including NSCLC[2,3,22] and the relative resistance of normal cells is thought to relate to the differential expression of TRAIL receptors[23] that may relate to p53 induced up-regulation of death receptors in response to DNA damage.[24]

We found that expression of DR5 was not only significantly higher in primary NSCLC, but also in neoplastic precursor lesions of squamous metaplasia and squamous CIS compared with normal bronchial epithelium, suggesting that alterations of DR5 occur early in the neoplastic process. Similar findings have been reported in precursor adenomatous lesions and carcinoma of the colon, compared with normal colonic mucosa.[18] In addition, a study of cervical cancer and precursor cervical intraepithelial neoplasia found a greater frequency of homogenous DR5 staining throughout the epithelium with increasing degrees of neoplasia.[20]

Studies of TRAIL receptors in high-stage NSCLC have reported high DR5 protein expression in 82% of stage III tumors[12] and only 32% of stage IIIB–IV tumors.[11] Although our experiments showed the majority of NSCLC cases had high expression of DR5, more stringent criteria were used to determine increased levels of expression. Comparison of different studies can be difficult because of the semiquantitative nature of immunohistochemical assessment and the lack of standardized methodology regarding criteria to determine positivity. Different antibodies

for detecting protein expression, differences in patient populations, and tumor stage may also contribute to the range of results.

Despite their roles as membrane-bound receptors, immunohistochemical expression of TRAIL receptors is characteristically cytoplasmic rather than membranous. Numerous investigators have found immunohistochemical expression of DR5 protein to be cytoplasmic in normal and neoplastic tissues[11,17–20,25,26] including lung carcinoma.[12,23] Similarly, other members of the TNF receptor family including Fas and TNF-R1 and R2 also show cytoplasmic expression and it has been postulated that TRAIL receptors may exist in a soluble, as well as membrane-bound form accounting for this finding.[18] Others have shown that TRAIL death receptors, Fas and TNF receptors are located in the Golgi apparatus and cell membrane before ligand exposure.[27–29]

DR5 is a proapoptotic receptor but the results of our study did not show a survival advantage for tumors with high expression of DR5. Instead, DR5 was found to be an adverse prognostic marker in univariate analyses, and was an independent predictor of disease-free survival among smokers, but not among the entire cohort of NSCLC after adjusting for other relevant covariates. DR5 was an adverse independent prognostic marker in studies of high-stage NSCLC[12] as well as breast carcinoma.[19] In melanomas, high DR5 expression was associated with a longer disease-free survival in univariate analysis but was not an independent prognostic factor in multivariate analysis.[17] Studies of DR5 in colon cancer,[26,30] ovarian cancer[25], and acute myelogenous leukemia[31] have not shown any association with prognosis. These findings are unexpected if death receptor-mediated proapoptotic signals are taking effect. Recently, several studies have shown that TRAIL death receptors can also activate signaling pathways that are antiapoptotic and hence could favor tumor growth.[32–34] DR5 has the potential to mediate antiapoptotic signals through activation of the nuclear factor κB (NF-κB) pathway[5,32,35] that is thought to play a role in tumorigenesis by inhibition of apoptosis. Recent studies have highlighted potential different roles of TRAIL receptors in different subcellular locations.[36–38] TRAIL death receptors undergo low levels of spontaneous endocytosis and upon binding TRAIL, the complexes are rapidly internalized with subsequent reduction in surface expression of death receptors.[29,36,38] Unlike other members of the TNF family, this process is not necessary for transmission of a proapoptotic signal and may even reduce apoptosis in some circumstances.[36,38] Others have found an association between location of death receptors and sensitivity to TRAIL-mediated apoptosis with cell surface expression required for transmission of an apoptotic signal.[37] Down-regulation of caspases[39] or up-regulation of antiapoptotic proteins involved in the intrinsic pathway such as Bcl-2 and Bcl-xL could also be involved in resistance to apoptosis.[1,40] FLIP (FLICE [Fas-associated death-domain-like IL-1β-converting enzyme]-inhibitory protein) competes with caspase 8 to bind the death-inducing signaling complex but cannot transmit the apoptotic signal[41] and is associated with resistance to TRAIL-induced apoptosis in a variety of tumors.[42] Although decoy receptors could contribute to a growth advantage and their overexpression correlates with TRAIL resistance in vitro,[5] there is evidence that these receptors do not protect cells from apoptosis under physiological conditions.[43]

In summary, we have shown that the TRAIL death receptor DR5 is involved in the development of NSCLC and is expressed at high levels in the majority of tumors but is not an independent predictor of prognosis. Further studies are needed to help establish the significance of DR5 expression in the development and progression of NSCLC and to determine any association with patient outcome under different treatment conditions.

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