The Expression of TUCAN, an Inhibitor of Apoptosis Protein, in Patients with Advanced Non-small Cell Lung Cancer Treated with Chemotherapy

AGNIESZKA CHECINSKA[1], JOOST J. OUDEJANS[2], SIMONE W. SPAN[1], JOSE A. RODRIGUEZ[1], FRANK A.E. KRUYT[1] and GIUSEPPE GIACCONE[1]

Departments of [1]Medical Oncology and [2]Pathology, VU University Medical Center, 1081 HV Amsterdam, The Netherlands

Abstract. *Background: TUCAN is a caspase recruitment domain (CARD)-containing protein involved in tumor biology by regulating apoptosis and the NFκB pathway. Inhibition of caspase-9 may cause drug resistance. The pattern of expression, localization and prognostic value of TUCAN in the tumors of patients with non-small cell lung cancer (NSCLC) treated with chemotherapy were assessed in this study. Materials and Methods: Using immunohistochemistry, the expression and localization of TUCAN was evaluated in forty-nine tumor specimens from patients with NSCLC who underwent neoadjuvant chemotherapy (32 stage IIB or IIIA), or palliative chemotherapy (17 stage IIIB or IV). The correlation between TUCAN expression and subcellular localization, major patient characteristics, response to the treatment and overall survival were assessed. Results: TUCAN expression was detectable in 34 out of 49 (69%) tumor specimens. Among the positively-stained specimens, three patterns of localization were observed: 5 samples (11%) showed exclusive nuclear localization, 13 samples (27%) contained only cytoplasmic staining and 15 (31%) showed both cytoplasmic and nuclear localization. There was no significant correlation between the localization of TUCAN and response to chemotherapy. Although TUCAN expression was not correlated with outcome, interestingly, exclusive cytoplasmic localization of TUCAN predicted shorter survival (p=0.027). Conclusion: Our results suggest that differential localization of TUCAN may be a prognostic factor for NSCLC, despite the lack of predictive value for response to chemotherapy.*

Lung cancer is the major cancer killer worldwide with an overall 5-year survival rate of less than 15% (1). Non-small cell lung cancer (NSCLC) represents approximately 80% of all cases of lung cancer and is often diagnosed at an advanced stage (2). The cornerstone therapy for NSCLC is surgery, but this is possible in, approximately, only 30% of cases. Patients with a more advanced stage are candidates for systemic chemotherapy, which, however, has limited efficacy. Resistance to chemotherapy is in fact common in NSCLC and is a major cause for treatment failure.

Chemotherapeutic agents and radiotherapy can trigger cancer cells by induction of programmed cell death, so-called apoptosis (3). The apoptotic cell death is negatively regulated by eight members of the anti-apoptotic inhibitor of the apoptosis (IAP) protein family, which up-regulation can lead to the development of drug resistance. Among the IAPs, XIAP, cIAP1 and cIAP2 are characterized by the presence of one to three copies of the baculoviral inhibitory repeat (BIR) domains (4). Some inhibitory proteins contain another regulatory domain, the caspase recruitment domain (CARD); TUCAN (Tumor-Up-regulated CARD-containing Antagonist of caspase-9) is one of these (5, 6). Both the BIR and the CARD motifs are involved in inhibition of initiator caspase-9 and effector caspases-3 and -7 of apoptosis, and may be responsible for drug resistance in cancer cells (4, 6).

We previously described a constitutive inhibition of caspase-9 in NSCLC (7). In search of a potential inhibitor, we screened a number of lung cancer cell lines. In this screening TUCAN was highly expressed in all NSCLC tested and absent in SCLC cell lines (8).

The CARD-containing inhibitory protein, TUCAN/CARDINAL, has dual activity as an inhibitor not only of

Abbreviations: NSCLC, non-small cell lung cancer; CARD, caspase recruitment domain; TNM, tumor-node-metastasis.

Correspondence to: G. Giaccone, MD, Ph.D., Department of Medical Oncology, VU University Medical Center, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands. Tel: +31-20-4444352, Fax: +31-20-4444079, e-mail: g.giaccone@vumc.nl

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apoptosis, but also of the NFκB pathway. CARDINAL potently suppresses NFκB activation associated with overexpression of TRAIL-R1, -R2, RIP, RICK, Bcl10 and TRADD (5, 9). TUCAN inhibits apoptosis by interfering with Apaf-1 binding to procaspase-9 *via* its CARD domain (5). TUCAN expression was shown to be correlated with poor prognosis in patients with stage II colon cancer treated with surgery alone, whilst overexpression of TUCAN correlated with shorter survival (5, 10).

The expression and localization of TUCAN in tumor samples from patients with advanced NSCLC treated with chemotherapy was examined. Although response to chemotherapy did not correlate with TUCAN expression, exclusive cytoplasmic localization of TUCAN was associated with significantly shorter survival. This may suggest that the different subcellular localization of TUCAN may have different prognostic implications.

Materials and Methods

Patients and specimens. Forty-nine patients with histologically documented NSCLC were included in this study. The major patient characteristics are summarized in Table I. The majority underwent neoadjuvant chemotherapy followed by surgery, while 17 patients received palliative chemotherapy alone. Patients were treated at the VU University Medical Center, Amsterdam, between January 1993 and December 1999. Chemotherapy was mainly platinum based and consisted of different regimens. Five patients, however, received a non-platinum doublet. The tissue samples were obtained from the surgical specimens or from diagnostic investigations. The most recent staging system was used (11). Response to chemotherapy was classified according to the World Health Organization (WHO) criteria (12).

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue was freshly cut, deparaffinized and rehydrated. Non-specific staining was blocked using 1:10 normal goat serum in PBS (pH 7.4)-1% BSA. The same anti-TUCAN antibody, previously used to detect TUCAN expression in colon cancer, was used (5). The specific rabbit polyclonal primary antibody Bur215, generated using a synthetic peptide with sequence corresponding to the residues 126-147 of TUCAN, was kindly provided by J.C. Reed (5); this was diluted 1:15,000 in blocking solution (1% BSA) and sections were incubated overnight at 4˚C in a humidified chamber. Sections were subsequently incubated for 30 min with HRP-conjugated secondary antibody and developed with diaminobenzidine tetrahydrochloride substrate (EnVision, DAKO, Glostrup, Denmark). As a positive control, colorectal carcinoma sections were used, as described by Pathan *et al.* (5). The omission of the primary antibody in simultaneously incubated sections was used as a negative control.

Statistical analysis. The association between TUCAN expression and selected patient or tumor characteristics was analyzed using the Chi-square test. Survival curves were constructed using the Kaplan-Meier method and compared using the log-rank test. A value of $p<0.05$ was considered statistically significant. Overall survival was calculated from the date of start of chemotherapy to the date of death, and progression-free survival was computed from the date of start of chemotherapy to the date of documented progression or death. Statistical analysis was performed using the SPSS software program 10.0 (SPSS Inc, Chicago, IL, USA).

Table I. *Patient characteristics.*

Total number	49
Age (years), median (range)	57 (29-75)
Gender	
male	32
female	17
Stage	
IIB	3
IIIA	26
IIIB	9
IV	11
Histology	
squamous	19
adenocarcinoma	20
large cell	10
Differentiation grade	
well	1
moderate	18
poor	30
Smoking history	
current smoker	22
non-smoker	3
ex-smoker	17
unknown	7
Prior therapy	
none	42
surgery	4
radiotherapy	3
Chemotherapy	
neoadjuvant	32
palliative	17
Response to chemotherapy	
yes	30
no	19

Results

Expression and localization of TUCAN. TUCAN expression was performed in a series of 49 paraffin-embedded NSCLC specimens. TUCAN expression was detectable in 34 (69%) tumor specimens and the neighboring normal lung epithelial tissue did not cross-react with the TUCAN antibody. The staining had a diffuse appearance and the staining intensity was scored on a scale from 0 to 3+.

TUCAN immunoreactivity was found in different subcellular compartments. Among the positively stained specimens 5 samples (11%) showed nuclear localization (Figure 1A), 13 samples (27%) contained only cells with cytoplasmic TUCAN (Figure 1B), while 15 (31%) showed cytoplasmic and nuclear TUCAN localization (Figure 1C).

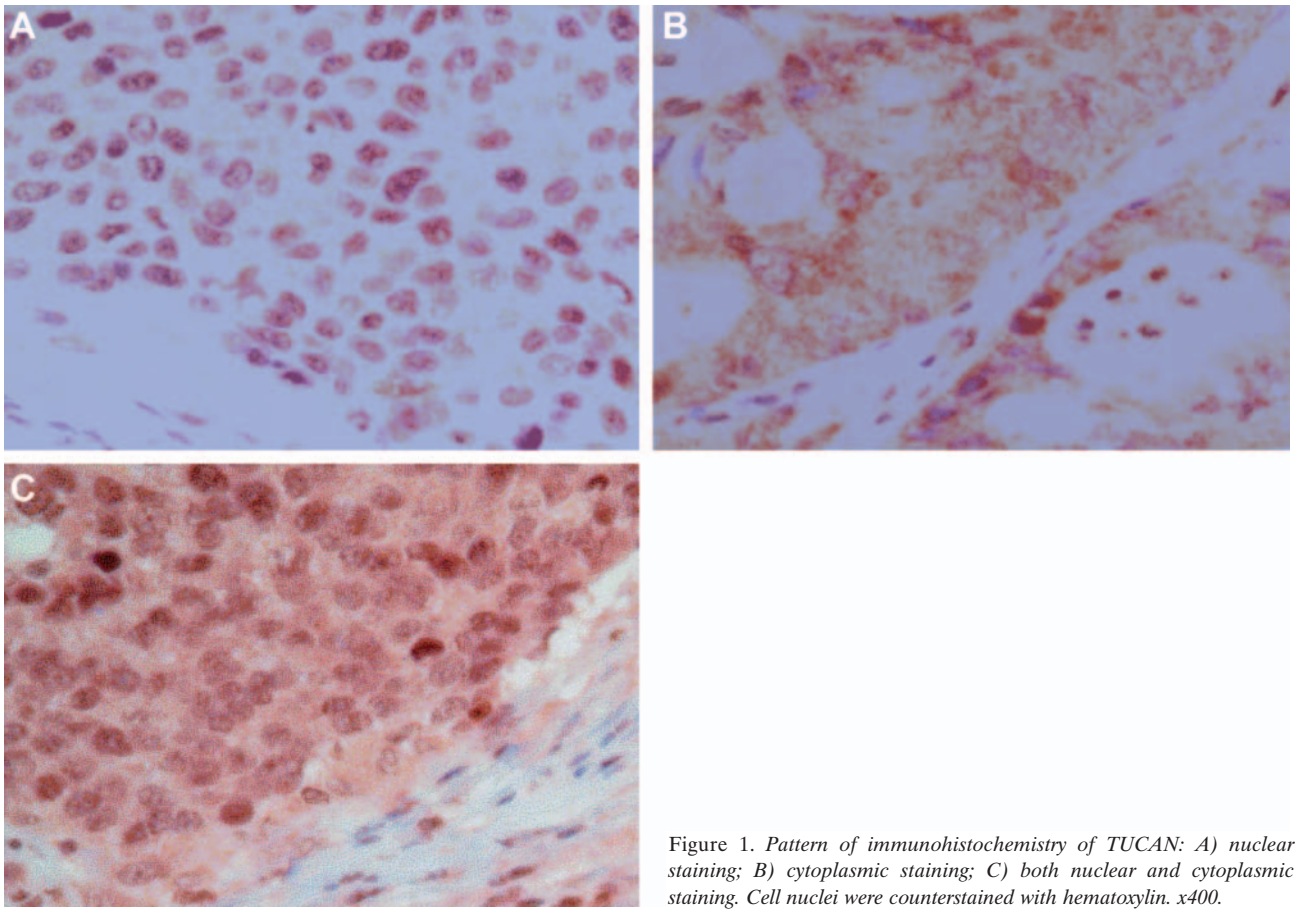

Figure 1. *Pattern of immunohistochemistry of TUCAN: A) nuclear staining; B) cytoplasmic staining; C) both nuclear and cytoplasmic staining. Cell nuclei were counterstained with hematoxylin. x400.*

We analyzed the impact of different localizations of TUCAN within the cell and identified groups based on the absence or presence of cytoplasmic TUCAN staining, nuclear staining, and the exclusive presence of cytoplasmic or nuclear staining.

The expression of TUCAN was significantly more frequent in men (27/32, 84% *vs.* 7/17, 41%; $p=0.003$) and in patients who received palliative chemotherapy (16/17, 94% *vs.* 18/32, 56%; $p=0.008$). Considering TUCAN localization and patient characteristics, male gender was also significantly associated with samples having cytoplasmic TUCAN ($p=0.035$) and in those with exclusive cytoplasmic TUCAN ($p=0.018$), whereas the type of chemotherapy lost significance in these subgroups.

The response to chemotherapy was 61.2%, relatively high, reflecting the fact that most patients received chemotherapy as induction for a relatively early stage. There was no correlation between response and TUCAN expression even when intensity of TUCAN staining was taken into consideration.

TUCAN expression, localization and survival. At the time of this analysis, the median follow-up was 8.5 years (range 1-148.5 months). Seven patients were reported alive and 42 dead. The median survival of all patients was 19.5 months and the 1- and 2-year survival rates were 76% and 37%, respectively. Median time to progression was 11 months. The only clinical factors that significantly influenced survival were stage (stage IIB+III 23 months median survival *vs.* 14 months for stage IV, $p=0.0051$), type of chemotherapy (neoadjuvant 22 months *vs.* 13 months for palliative, $p=0.0147$) and response to chemotherapy (response 24 months *vs.* 13 months no-response, $p=0.0046$).

The presence of TUCAN staining and the intensity of staining did not have any impact on survival (Table II). Interestingly, however, the presence of cytoplasmic staining was associated with a poorer survival compared with the cases where no cytoplasmic staining was present (median survivals of 16 *vs.* 28 months, respectively, $p=0.154$) (Figure 2A). When exclusive cytoplasmic staining was considered (excluding the cases where both cytoplasmic and nuclear staining were present), this difference was statistically significant (median survival of 13 *vs.* 22 months, respectively, $p=0.027$) (Figure 2B).

The differences in subcellular localization prompted us to investigate TUCAN for the presence of potential nuclear

Table II. *Survival rates.*

Factor	n	Median survival (months)	*p*-value
Gender			
male	32	16	
female	17	24	0.076
Age			
>57	26	18	
<57	23	21	0.417
Response			
yes (CR+PR)	30	24	
no (NC+PD)	19	13	0.0046
Histology			
squamous	19	20	
adenocarcinoma	20	17	
large cell	10	18	0.073
Stage			
IIB	3		
III	35 (26IIIA+ 9IIIB)	23 (survival of II+III)	
IV	11	14	0.0051
Differentiation			
well+moderate	1+18	20	
poorly	30	18	0.37
Chemotherapy			
platinum	44	20	
non-platinum	5	13	0.57
Chemotherapy			
neoadjuvant	32	22	
palliative	17	13	0.0147
Smoking history			
smoker	22	17	
never-smoker	3	24	
ex-smoker	17	28	
unknown	7	16	0.070
TUCAN			
yes	34	17	
no	15	22	0.215
TUCAN nuclear			
yes	20	20	
no	29	18	0.568
TUCAN cytoplasmatic			
yes	28	16	
no	21	28	0.154
Exclusive TUCAN			
only nuclear	5	34	
other	44	18	0.989
Exclusive TUCAN			
only cytoplasmic	14	13	
other	35	22	0.027
TUCAN			
low (0-1)	30	18	
high (2-3)	19	20	0.473

export sequences (NESs) or nuclear localization signals (NLSs). Using computer-assisted analysis with the PSORT II and NetNES programs (13, 14), no NLS and only one potential NES was identified in the amino-acid sequence of TUCAN. Experimentally, the nuclear-cytoplasmic shuttling potential of TUCAN was tested in two ways: first, by transfection of a TUCAN-YFP construct in MCF7 cells and treatment with leptomycin B (LMB), a specific inhibitor of the nuclear export receptor CRM1, which causes a nuclear accumulation of NES-containing proteins (15); second, by co-expression of TUCAN-Flag with YFP-CRM1 in MCF7 cells. In neither experiment was any significant change in the nucleo-cytoplasmic localization of TUCAN observed (data not shown), suggesting that CRMI does not play a role in determining the nucleocytoplasmic distribution of TUCAN.

Discussion

TUCAN has been identified in normal tissues and in several tumor types. The presence of TUCAN in cancer cells may be responsible for increased aggressiveness of the tumor, by negative regulation of programmed cell death (5). TUCAN may inhibit caspase-9 and might be potentially responsible for drug resistance. In this study, we investigated the expression or localization of TUCAN in patients with NSCLC who underwent chemotherapy.

The majority of NSCLC samples express TUCAN, which is localized either exclusively in the nucleus (11%), or the cytoplasm (27%), or in both compartments (31%). Although we did not observe a correlation of TUCAN expression or localization with response to chemotherapy, patients whose tumors had TUCAN expression in the cytoplasm had a poorer survival and this was statistically significant when exclusive TUCAN expression in the cytoplasm was considered.

The heterogeneous subcellular localization of TUCAN in clinical samples and the correlation of cytoplasmic TUCAN with poor patient prognosis led us to investigate the mechanisms that may account for its presence in the cytoplasm. Our preclinical data suggest that cytoplasmic localization of TUCAN is not determined by CRM1-mediated export and is, therefore, not actively exported from the nucleus. Other, yet to be elucidated mechanisms, are apparently involved in determining the subcellular localization of TUCAN.

The level of TUCAN expression and different localization patterns did not predict the response to chemotherapy. These results are consistent with previous observations, where other IAPs (cIAP1, cIAP2, XIAP and survivin) failed to predict response to chemotherapy in NSCLC samples (16, 17). Interestingly, XIAP was also shown to have prognostic value in patients with radically resected NSCLC, despite the fact that it has not being predictive of response to chemotherapy (18), suggesting a role of some IAPs in the biology of NSCLC, rather than sensitivity to anticancer drugs. Furthermore, localization of survivin also was shown to be important for the prognosis of radically resected NSCLC (17).

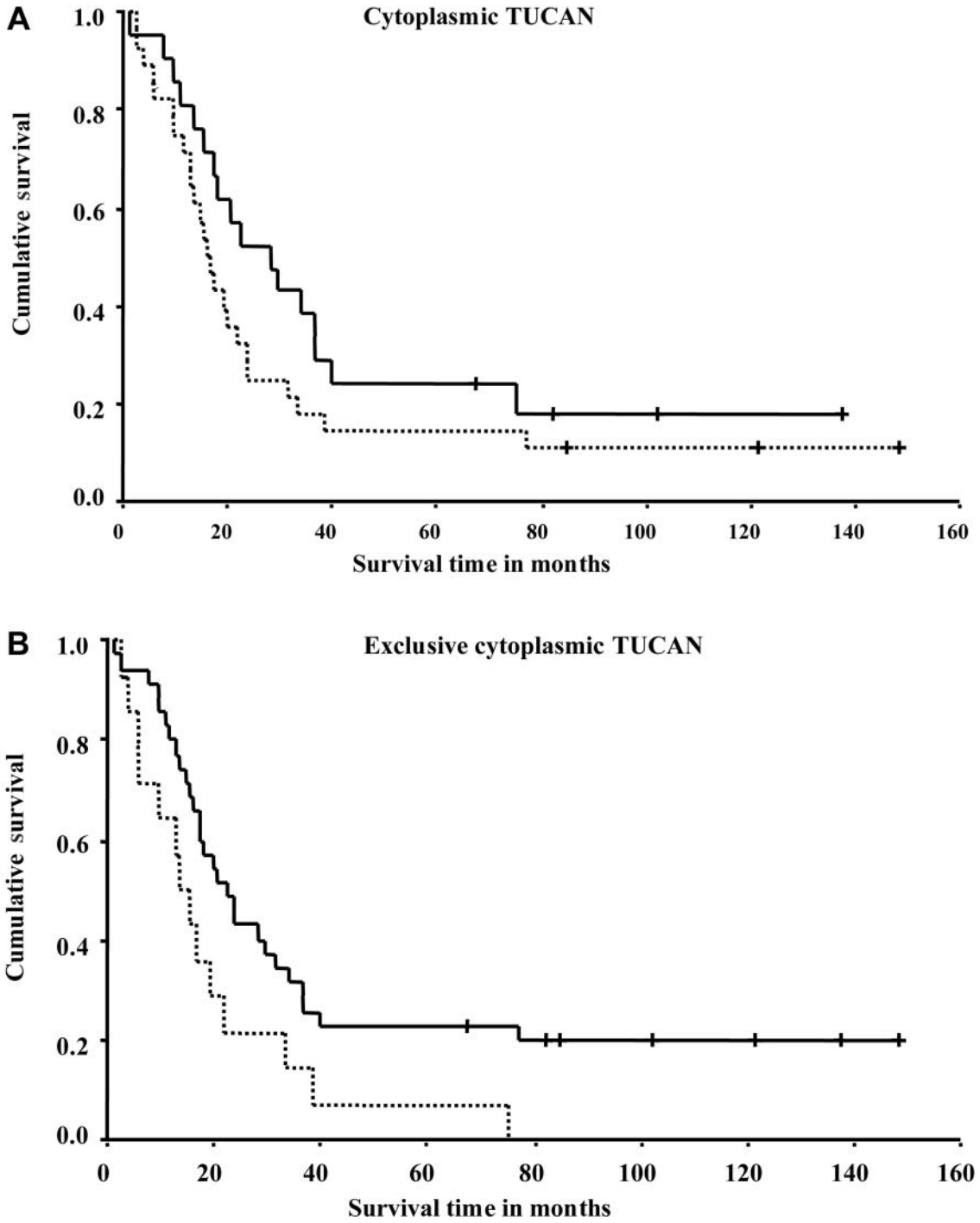

Figure 2. *A) Survival curves of patients with cytoplasmic TUCAN (dotted line) vs. patients with other patterns of TUCAN expression (solid line). Log-rank test, p=0.154; B) Survival curves of patients with exclusive cytoplasmic TUCAN (dotted line) vs. non-cytoplasmic TUCAN (solid line) expression patterns. Log-rank test, p=0.027.*

Interestingly, in a study performed in 102 specimens of patients with stage II colon carcinoma who only were treated with surgery, TUCAN was shown to be present in approximately two thirds of cases and its expression was primarily present in the cytosol and a high level of expression of TUCAN correlated with shorter overall survival in this cohort (5). The same series was later assessed for the expression of other apoptosis related genes

and high levels of TUCAN expression indicated higher risk of death using multivariate analysis (10). Moreover, in the group of patients that expressed low level of Apaf-1 and high level of TUCAN, only 37% of patients remained alive *versus* 83% of other patients after 8 years of follow-up (10).

In conclusion, this study demonstrated that although TUCAN expression was not correlated with response to chemotherapy in NSCLC patients, exclusive cytoplasmic expression of TUCAN appeared to be a prognostic factor. This finding should be confirmed in a larger sample size of radically-resected patients with NSCLC.

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