SPANDIDOS PUBLICATIONS

Tumour necrosis factor-α: Prognostic role and relationship with interleukin-8 and endothelin-1 in non-small cell lung cancer

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Abstract. Cytokines mediate numerous physiological and immune reactions, which are manifested in various biological effects, including tumouricidal activity. We evaluated the expression of the pleiotropic cytokine, tumour necrosis factor-α (TNF-α), by competitive PCR technique in 47 non-small cell lung cancer (NSCLC) cases and the impact of TNF-α on their clinical behaviour. Using univariate analysis, our study demonstrated a positive correlation between high TNF-α expression and favourable prognosis in NSCLC in terms of overall survival and disease free interval (p=0.03 and 0.04, respectively) and TNF-α maintained its independent role in multivariate analysis. TNF-α can stimulate the expression of many molecules, including interleukin-8 (IL-8) and endothelin-1 (ET-1); in our study, the expression of TNF-α was significantly associated with high IL-8 mRNA levels (p=0.008) and ET-1 mRNA positivity (p=0.03). We suggested that TNF-α can induce ET-1 mRNA expression in NSCLC, similarly to IL-8 expression. Our study may also contribute to advancing the knowledge of the molecular relationship between cytokines and endothelial functions in NSCLC.

Introduction

Lung cancer is the most common malignant disease occurring in males and the second most common malignant disease occurring in females. Despite surgical treatment, chemotherapy and radiotherapy, the rate of 5-year survival is minimal: in fact, it does not exceed 15% in non-small cell lung cancer, and 2% in small cell lung cancer (1).

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Abbreviations: TNF-α, tumour necrosis factor-α; IL-8, interleukin-8; ET-1, endothelin-1; NSCLC, non-small cell lung cancer; RT-PCR, reverse transcriptase-polymerase chain reaction

Key words: tumour necrosis factor-α, interleukin-8, endothelin-1, non-small cell lung cancer, angiogenesis

The occurrence of a large number of tumours in immunocompromised patients indicates a role of the immune system in tumour growth. The significance of immune surveillance in tumours, and more specifically in lung cancer, has still not been sufficiently studied. Both immunocompetent cells and tumour cells secrete many cytokines which have an important role in the stimulation of cellular activity. Cytokines mediate numerous physiological and immune reactions, which are manifested in various biological effects, including tumouricidal activity.

Tumour necrosis factor-α (TNF-α) is a pleiotropic cytokine which can be either constitutively produced or induced in human tumours, and experimental studies underline the involvement of TNF-α in processes which contribute to cancer progression (2). *In vitro* studies have shown an antiproliferative effect of TNF-α against various non-small cell lung cancer (NSCLC) cell lines (3). In addition, TNF-α can stimulate the expression of many molecules, including interleukin-8 (IL-8) and endothelin-1 (ET-1); however, the combined use of interleukin and TNF-α in clinical trials of patients with advanced NSCLC has demonstrated both conflicting and disappointing results. The aim of this study was to analyse the effect of TNF-α on IL-8 and endothelin-1, in order to clarify the mechanisms of TNF-α-induced gene expression in NSCLC. Moreover, the impact of TNF-α on the clinical behaviour of patients was also evaluated.

Materials and methods

Surgical specimens. Forty-seven NSCLC patients, who had undergone curative surgical resection at the Department of Cardio-Thoracic Surgery, University of Pisa, between 1991 and 1994, were analysed. There were 37 males and 10 females (mean age 63.2 years, median 64 years, range 42-77 years). All of the patients underwent a complete preoperative staging. The most common histological type was squamous carcinoma (23 cases), followed by adenocarcinoma (16 cases), large-cell anaplastic carcinoma (4 cases) and bronchiolo-alveolar carcinoma (4 cases). Tumours were classified according to the World Health Organization Classification (4) and according to the guidelines of the American Joint Committee for Cancer Staging (5). According to tumour-status, tumours were classified as follows: 8 T1, 34 T2, 5 T3; twenty-nine patients

did not show nodal metastasis at the moment of diagnosis, whereas 18 showed hilar and/or mediastinal metastatic involvement. Data on clinical behaviour were available in all 47 cases (median follow-up 133 months, mean 125.1 months, range 75-146). Twenty-one patients were alive at the end of follow-up, whereas 26 had died. Tumour samples were partly frozen in liquid nitrogen and stored at -80˚C for molecular studies and partly formalin-fixed and paraffin-embedded for histological processing.

RNA extraction. Total RNA was extracted from frozen lung tissue samples using RNeasy Mini Kit (Qiagen, M-Medical s.r.l., Florence, Italy); high-quality RNA was then eluted in 30 μl of water. Purified RNA was digested with RNase-free DNase to guarantee that the RNA was completely free of DNA contamination.

RT-PCR analysis. A constant amount of total RNA (5 μg) was reverse-transcribed at 42˚C for 60 min in a total 20-μl reaction volume using a First-Strand cDNA synthesis kit for RT-PCR (AMV) (Roche Diagnostic Corporation, Indianapolis, IN, USA). cDNA was incubated at 95˚C for 5 min to inactivate the reverse transcriptase and served as a template DNA for amplification using the Gene Amp PCR System 2400 (Perkin-Elmer Applied Biosystem, CA). PCR was performed in a standard 50-μl reaction mixture consisting of 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ (pH 8.3), 0.2 mM dNTPs, 50 picomoles of each sense and antisense primer, and 2.5 U of AmpliTaq DNA Polymerase (EuroBioTaq, Eurobio, Les Ulis, France). As negative control, the DNA template was omitted in the reaction.

PCR primers for TNF-α cDNA were as follows: forward primer, 5'-GAGTGACAAGCCGTAGCCCATGTTGTAG CA-3'; reverse primer, 5'-GCAATGATCCCAAAGTAGAC CTGCCCAGACT-3'. Amplification was performed for 45 sec at 94˚C, 45 sec at 60˚C and 2 min at 72˚C for 35 cycles; lastly, an additional extension step was performed for 7 min. A single 444-bp band was detected.

PCR primers for IL-8 cDNA were as follows: forward primer, 5'-ATGACTTCCAAGCTGGCCGTGGCT-3'; reverse primer, 5'-TCTCAGCCCTCTTCAAAAACTTCTC-3' (Clontech Laboratories, Inc., Palo Alto, CA). Amplification was performed for 45 sec at 94˚C, 45 sec at 60˚C and 2 min at 72˚C for 35 cycles; an additional extension step was performed for 7 min. Amplified PCR products were run on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. A single 289-bp band was detected.

PCR primers for ET-1 cDNA were 5'-TTGAGATCTG AGGAACCC-3' and 5'-TACGGAACAACGTGCTCG-3' (Clontech Laboratories, Inc.). Amplification was performed for 30 sec at 95˚C, 30 sec at 58˚C and 30 sec at 72˚C for 30 cycles, and an additional extension step of 10 min. A single 354-bp band was detected.

The presence of a 412-bp band, amplified with primer specific for GAPDH with the same cDNAs, was used as internal control (6). No band was detected when no cDNA was added to the PCR mixture.

Quantitative PCR reaction. In order to obtain the quantitation of TNF-α and IL-8 mRNA levels, we used a technique based on a competitive PCR approach employing a non-homologous internal standard called PCR MIMICs (Clontech). This method, previously described by Boldrini *et al* (7), involves amplification of a heterologous DNA fragment (BamHI/EcoRI 574-bp fragment of v-erbB) with a pair of composite primers, which contain the target primer sequences contiguous to a sequence that anneals the heterologous DNA fragment. During amplification, the target primer sequences were incorporated into the products. Thus, we refer to this heterologous competitor fragment as Competitor because it competes with the target gene for primer annealing and amplification. Known amounts of Competitor (600-bp for TNF-α and 400-bp for IL-8,) were added to aliquots of cDNA derived from 5 μg of total RNA. The relative densitometric measure of the electrophoretic bands was then plotted, and the point of equal intensity between the bands of Competitor PCR MIMIC and TNF-α or IL-8 was taken as concentration of the cDNA sample.

Statistical analysis. All statistical analyses were carried out using Statistica software (Stat-soft). Univariate analysis was performed by modeling Kaplan-Meier survival curves. The log-rank test was used to evaluate the statistical significance of differences in survival distributions among prognostic

Table I. Univariate survival analysis.

Features	No. of patients	Overall survival p-value[a]	Disease-free interval p-value[a]
Sex			
Male	37	0.52	0.47
Female	10		
Age			
≤ 64	26	0.38	0.44
>64	21		
Histology			
Squamous	23	0.9	0.95
Adeno	16		
Anaplastic	4		
Br. alveolar	4		
Tumour status			
T1	8	0.28	0.5
T2	34		
T3	5		
Node status			
N0	29	0.02	**0.02**
N1	5		
N2	13		
TNF-α			
Low	37	**0.03**	**0.04**
High	10		

[a]Log-rank test.

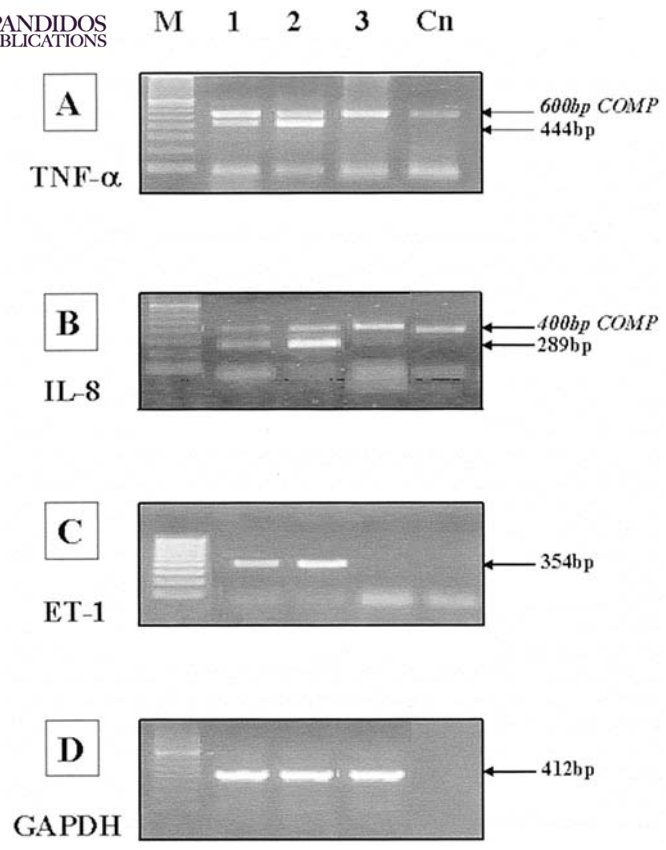

Figure 1. A and B show, respectively, competitive PCR analysis of relative TNF-α and IL-8 mRNA levels. Lane 1, NSCLC with higher ratio between Competitor and both TNF-α and IL-8; lane 2, NSCLC with lower ratio between Competitor and both TNF-α and IL-8; lane 3, negative sample, showing only the Competitor band; Cn, negative control with no cDNA; M, marker (100-bp ladder, Pharmacia). C shows an example of RT-PCR for ET-1; lanes 1 and 2, two positive NSCLC samples with moderate and high levels of ET-1 mRNA expression; lane 3, a negative sample; Cn, negative control with no cDNA; M, marker (100-bp ladder, Pharmacia). D, amplification of the housekeeping GAPDH gene (412 bp) is shown in the same samples.

groups. Multivariate analysis was performed to evaluate the independent prognostic role of TNF-α expression after accounting for other covariates. The *a priori* level of significance was set at p-value <0.05.

Results

Clinicopathological parameters and survival. Following WHO and AJCCS criteria, 8 of the 47 (17%) NSCLC samples were classified as T1, 34 (72.3%) as T2 and 5 (10.7%) as T3. Eighteen cases (5 N1 and 13 N2) showed nodal metastatic involvement at diagnosis. Among the clinicopathological parameters analysed, the absence of metastatic nodal involvement was significantly associated with better overall survival (p=0.02) and longer disease-free interval (p=0.02) (Table I).

TNF-α expression. TNF-α mRNA expression, evaluated by PCR technique, was observed in 27 of the 47 cases (57.44%); Fig. 1A shows the results of the electrophoretic analysis of PCR products in representative cases.

We performed competitive PCR reactions with a known amount of Competitor and cDNA derived from a fixed amount of total RNA. The amount of TNF-α cDNA molecules was calculated by extrapolating from the point of equal signal of Competitor and target. Assuming a median value of 340 cDNA molecules for TNF-α expression as the cut-off value, we distinguished between tumours with low and high TNF-α messenger levels. Low TNF-α mRNA expression (≤ median value) was detected in 37 NSCLC samples, while high TNF-α mRNA levels were found in 10 cases.

Table II. Cox proportional-hazard model of overall survival.

Variables	Beta	Standard error of beta	t-test	p-value
Tumour status	-0.177	0.153	-1.158	0.253
Node status	-0.275	0.14	-1.952	**0.048**
Histotype	0.137	0.142	0.965	0.339
TNF-α	0.341	0.153	2.22	**0.031**

Association of TNF-α with clinicopathological characteristics and survival. Using univariate analysis, TNF-α was significantly associated with a favourable prognosis in terms of overall survival and disease free interval (p=0.03 and 0.04, respectively) (Table I). Fig. 2 shows Kaplan-Meier survival curves generated on the basis of low and high expression of TNF-α mRNA.

Multivariate analysis. After demonstrating that TNF-α significantly influenced survival at univariate analysis, a Cox proportional-hazard model showed that TNF-α expression and nodal status maintained their independent prognostic roles after accounting for other covariates (Table II).

IL-8 expression. IL-8 expression, evaluated by PCR technique, was observed in 44 of the 47 (93.6%) NSCLC cases. We performed competitive PCR reactions with a known amount of Competitor and cDNA derived from a fixed amount of total RNA. A representative electrophoretic analysis of competitive PCR products for IL-8 appears in Fig. 1B. The amount of IL-8 cDNA molecules was calculated by extrapolating from the point of equal signal of Competitor and target IL-8. Assuming a median value of 350 cDNA molecules for IL-8 expression as the cut-off value, we distinguished between tumours with low (20 cases, 42.5%) and high IL-8 messenger levels (27 cases, 57.5%).

Relationship between TNF-α and IL-8 mRNA expression. A high expression of TNF-α was significantly associated with high IL-8 mRNA levels; tumours with high TNF-α expression showed a higher number of IL-8 cDNA molecules than samples with low TNF-α expression (t-test; p=0.008) (Table III).

ET-1 mRNAs in NSCLC. In the limited number of cases in which the material was available (37 NSCLC samples), ET-1 mRNA expression was also evaluated by RT-PCR (Fig. 1C). Sixteen of the 37 patients (43.24 %) showed positivity for endothelin mRNA.

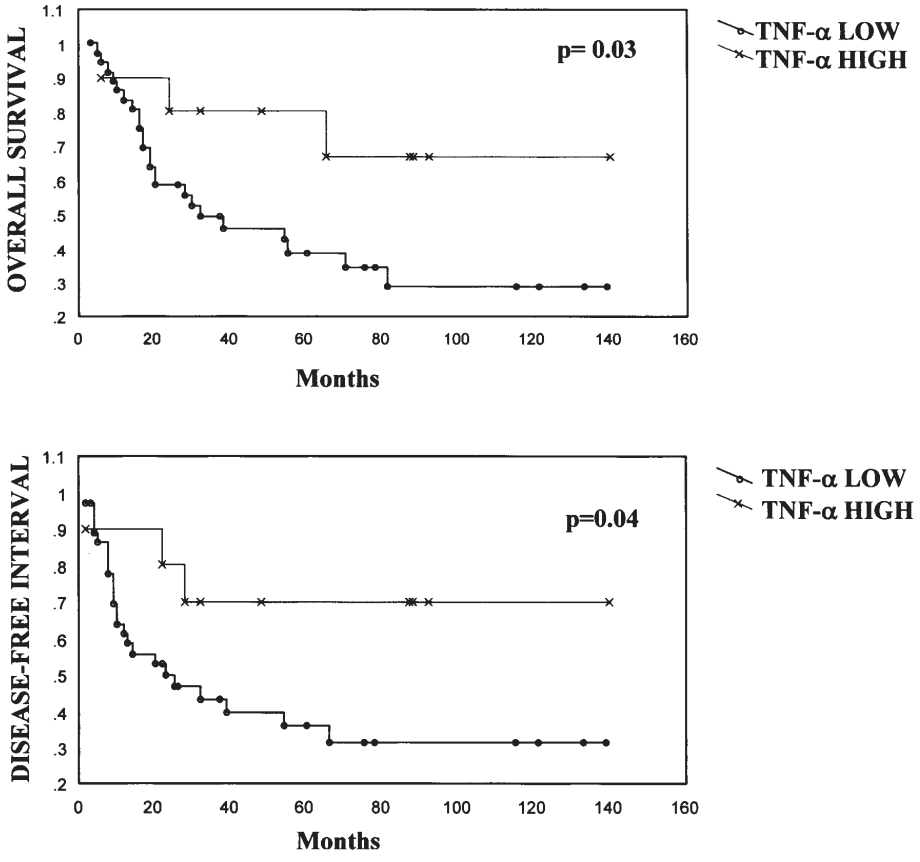

Figure 2. Kaplan-Meier overall survival and disease-free interval curves in relation to TNF-α mRNA expression levels in NSCLC samples.

Table III. Relationship between TNF-α and IL-8 mRNA expression levels.

	TNF-α		
	Low	High	p-value
IL-8 cDNAmolecules			
Mean ± SD	422.9±499	951±662	**0.008**

Table IV. Relationship between TNF-α expression and ET-1 mRNA expression.

	TNF-α		
	Positive	Negative	p-value
ET-1			
Positive	12	4	**0.03**
Negative	8	13	

Relationship between TNF-α and endothelin mRNA expression. Among the 37 NSCLC samples analysed for endothelin expression, positivity for ET-1 mRNA was associated with the expression of TNF-α messenger (Table IV), suggesting that ET-1 mRNA expression may be induced by TNF-α similarly to IL-8.

Discussion

Cytokines mediate several physiological reactions, and recent *in vivo* and *in vitro* studies have demonstrated a wide spectrum of biological activities of cytokines in the pathogenesis and progression of malignancies (8-11). TNF-α has emerged as one of the many mediators that, either alone or in combination, appears to mediate both antiproliferative and tumourigenic effects in malignant tumours; in fact, the association between TNF-α expression and tumour prognosis has been variable. An antiproliferative effect of TNF-α has been demonstrated in various malignancies, such as colon (12,13) and kidney (14) carcinomas, as well as malignant melanoma (15). In contrast, a protumourigenic effect of TNF-α has been reported in ovarian epithelial carcinoma (16) and breast carcinoma (17).

Several studies have reported an antiproliferative effect of TNF in NSCLCs. Munker *et al* (18) displayed an inhibitory role of TNF-α in clonal proliferation of all nine NSCLC cell lines examined. Other *in vitro* studies (19,20) with established cell lines or with single cell suspensions from fresh NSCLC tissue also showed, albeit to a lesser degree, an antitumourigenic effect of TNF in NSCLC. Tran *et al* (3) showed an association between TNFs and a better clinical outcome in 71 NSCLC patients. However, despite promising results from *in vitro* and *in vivo* studies, clinical trials using TNF-α in combination with other cytokines for the treatment of patients

anced NSCLC have yielded disappointing and g results, and more data are needed concerning the prognostic role of TNF-α.

Using univariate analysis, our study demonstrated a positive correlation between high TNF-α expression and favourable prognosis in NSCLC, and TNF-α maintained its independent role with multivariate analysis. The favourable effect on clinical outcome may be due to the ability of TNF-α to cause haemorrhagic necrosis of the tumour; the endothelial cells of tumour blood vessels may represent target cells for the cytotoxic effect of TNF-α (21), with a consequent tumouricidal activity of this cytokine.

Moreover, apart from direct cytotoxicity, TNF-α can also indirectly cause tissue damage through synergistic action with other cytokines and/or through interaction with other molecules.

IL-8 is an important cytokine involved in proinflammatory and reparative processes (22), and it is widely expressed in tumoural, stromal and endothelial cells (23-27). In our previous study (28), a high expression of IL-8 mRNA was significantly associated with a high vascular density and a high expression of VEGF mRNA. Based on these data, we supposed a key role of IL-8 in angiogenesis, although we hypothesized that IL-8 and VEGF mRNA were up-regulated by different pathways, as affirmed by Yuan *et al* (29).

In this study, we analysed IL-8 mRNA expression in 47 NSCLC samples, with particular attention being given to correlation with TNF-α mRNA expression. IL-8 mRNA seems to be induced by TNF-α in epithelial cells (30), in leukocytes (31) and in lung cancer cells (32). Our data confirmed high IL-8 expression in NSCLC samples showing high TNF-α levels, indicating that tumour-derived IL-8 may be important in recruiting inflammatory neutrophils and in promoting interactions between inflammatory cells and lung cancer cells; this may result in an association of high IL-8 levels either with tumour progression or regression (33,34).

Endothelins are peptides encoded by distinctive genes ET-1, ET-2, ET-3 and ET-4 (35). They are converted by endothelin-converting enzymes (ECE) from 'big endothelins' originating in large preproendothelin peptides cleaved by endopeptidases (36). ET-1, a 21-amino-acid peptide, has a major influence on cell proliferation through activation of specific ET-A and ET-B receptors (37). A possible involvement of ET-1 expression in lung tumour development has been recently suggested (38), even if several aspects of the potential role of ET remain unclear. Several cytokines can stimulate endothelin; in this study, we suggest that TNF-α can induce ET-1 mRNA expression in NSCLC, similarly to IL-8 expression. However, the signal transduction pathway of TNF-α-induced ET-1 expression may be different from that of TNF-α-induced IL-8 expression in NSCLC; moreover, ET-1 may have a role only in the specific status of lung cancer stroma, as suggested by Finsnes *et al* (39), who demonstrated ET-1 production in association with eosinophilic rather than neutrophilic airway inflammation.

This study may contribute to advancing the knowledge of the molecular relationship between cytokines and endothelial functions in NSCLC. However, further studies are necessary to completely understand the molecular mechanisms of the prognostic effect of TNF-α. Moreover, polymorphisms in the promoter regions of cytokine genes are associated with differential levels of cytokine expression and might influence tumour development and progression by affecting the efficiency of the antitumour immune response and/or angiogenesis pathways (40-43); investigations are required to establish the possible role of cytokines, including TNF-α, and gene polymorphisms in both the susceptibility to and prognosis of NSCLC.

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