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Original Paper

Bcl2 and p53 Regulate Vascular Endothelial Growth Factor (VEGF)-mediated Angiogenesis in Non-small Cell Lung Carcinoma

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The aim of this study was to investigate the expression of p53 and bcl2 proteins in a series of 107 non-small cell lung cancers (NSCLC), and to relate such protein expression to neovascularisation and the expression of vascular endothelial growth factor (VEGF). Moreover, we analysed the prognostic impact of these biological parameters on overall survival, both in univariate and multivariate analyses. An inverse association was found between bcl2 expression and microvessel count (MVC; $P = 0.0004$) and bcl2 and VEGF ($P = 0.007$). In contrast, a significant association was found between p53 expression and MVC ($P = 0.03$) and p53 and VEGF expression ($P = 0.04$). In univariate analysis, nodal status ($P < 0.000001$), MVC ($P < 0.000001$), bcl2 ($P = 0.002$), p53 ($P = 0.03$) and VEGF expression ($P < 0.000001$) significantly affected overall survival, but in multivariate analysis only MVC and VEGF expression retained their prognostic influence. Our results suggest that bcl2 and p53 possibly control the development of tumour angiogenesis in NSCLC, with putative mediation by VEGF. Moreover, the important influence of angiogenesis in the progression of NSCLC is further highlighted.

Key words: bcl2, p53, angiogenesis, VEGF
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INTRODUCTION

TUMOUR GROWTH and progression strictly depend on an imbalance between cellular proliferation and death. Alterations in different genes, variously controlling the proliferation/death balance, are frequently involved in the development and progression of several types of human cancers [1]. Moreover, the growth of solid tumours beyond a certain critical mass and the development of tumour metastasis have been shown to be related to the establishment of a vasculature that provides continuous exposure to blood flow [2].

In non-small cell lung cancer (NSCLC), which represents the most common cause of cancer mortality in women and men, some molecular factors (i.e. proto-oncogenes and tumour suppressor genes) [3–6], as well as markers of metastatic propensity (i.e. tumour angiogenesis) [7–9], have been shown to be involved in the development and progression of this type of cancer, although the biological mechanisms underlining their involvement have not yet been completely clarified.

Recently, various experimental results have shown that some tumour suppressor genes are involved in the regulation of angiogenesis [10], and interesting data have been reported on the putative upregulation of an angiogenic specific stimulator (vascular endothelial growth factor (VEGF)) by a mutant form of p53 [11]. As p53 has been shown to be associated with VEGF in NSCLC [12] and since bcl2 and neoangiogenesis have been associated with a better and a worse prognosis, respectively [13], we carried out a study in order to clarify the role of p53 and bcl2 in angiogenesis in NSCLC.

PATIENTS AND METHODS

Patients and tumour tissues

107 NSCLC patients who had undergone curative surgical resection at the Department of Surgery, University of Pisa, between March 1991 and March 1992, were analysed. The

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median follow-up period was 51 months (range 28–59 months). There were 96 males and 11 females, with a mean age of 63.1 ± 7.2 years (range 46–81 years, median 64 years). The patients had no detectable metastases in distant organs at the time of surgery. They had not received either chemotherapy or radiation therapy before or after surgery. Tumour samples were in part frozen in liquid nitrogen and stored at −80° and in part formalin-fixed and paraffin-embedded for histological and immunohistochemical processing. Tumours were classified according to the World Health Organization classification [14] and according to the guidelines of the American joint Committee for Cancer Staging [15].

Immunohistochemistry and immunostaining evaluation

Microvessel detection and counting. The method of microvessel detection and counting has been described previously [8]. Briefly, intratumour microvessels were highlighted with anti-FVIII monoclonal antibody (MAb) (Dako, Santa Barbara, California, U.S.A.) diluted 1:50, overnight. Biotinylated antimouse IgG (Vector, Burlingame, California, U.S.A.) was then applied, followed by detection using the avidin–biotin peroxidase complex (ABC) method. A single microvessel was defined as any brown immunostained endothelial cell separated from adjacent microvessels, tumour cells and other connective tissue elements. Each sample was examined under low power (×10 objective lens and ×10 ocular lens) to identify the three regions of the section with the highest number of microvessels. A ×250 field (×25 objective lens and ×10 ocular lens; 0.74 mm^2 per field) in each of these three areas was counted and the average counts of the three fields were recorded. Large vessels with thick muscular walls were excluded in the counts. A lumen was not required to identify a vessel.

VEGF expression. Immunostaining for VEGF was performed using the ABC method. Sections were dewaxed in xylene, taken through ethanol and then incubated with 0.3% hydrogen peroxide in methanol for 10 min to block the endogenous peroxidase activity. After washing with phosphate buffered saline (PBS) and incubation with 10% normal goat serum, the sections were incubated overnight with an anti-VEGF polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California, U.S.A.), diluted 1:50. This antibody is raised against a synthetic peptide corresponding to amino acid residues 1–191 of human VEGF. It recognises the 165, 189 and 121 amino splicing variants of VEGF. After the primary antibody, biotinylated antirabbit IgG (Vector) was applied and followed by detection using the ABC method. Diaminobenzidine was used as the chromogen. Light counterstaining was performed with haematoxylin. Negative controls were obtained using PBS instead of the primary antibody. VEGF expression was evaluated as a percentage of positive cells in a total of at least 1000 tumour cells. Tumour sections with no VEGF immunoreactive cells were considered as negative. The median value of this series (40% of positive cells) was used as the cut-off value to distinguish tumours with low VEGF expression from tumours with high VEGF expression.

VEGF mRNA expression by reverse transcription–polymerase chain reaction (RT–PCR). Total RNA was extracted from frozen lung tissue using an RNA extraction reagent, ULTRASPEC™ RNA, according to the standard acid–guanidium–phenol–chloroform method. Five micrograms of total RNA were reverse-transcribed at 42°C for 60 min in a total 20 µl reaction volume using a 1st-strand™ cDNA Synthesis Kit (Clontech Laboratories, Palo Alto, California, U.S.A.). cDNA was incubated at 95°C for 5 min to inactivate the reverse transcriptase, and served as template DNA for 30 rounds of amplification using the Gene Amp PCR System 2400 (Perkin-Elmer Applied Biosystems, California, U.S.A.). 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 pmol of each sense and antisense primer, and 2.5 U of Amplitaq DNA Polymerase (Perkin-Elmer Applied Biosystems). Amplification was performed for 30 sec at 94°C, 1 min at 55°C and 1 min at 72°C. Finally, an additional extension step was carried out for 2 min. As a negative control the DNA template was omitted in the reaction. The amplification products were separated on 1.5% agarose gels and visualised by ethidium bromide staining. PCR primers for VEGF cDNA were as follows: forward primer, 5′-TGGATCCATGAACTTTCTGCTGTC-3′, reverse primer, 5′-TCACCGCCTTGGCTTGTCACAT-3′, according to the VEGF gene structure [1]. Three kinds of PCR product of 656, 584 and 452 bp encoding VEGF isoforms VEGF 189, VEGF 165 and VEGF 121, respectively, were obtained. For GAPDH, the forward primer was 5′-CGATGCTGGCGCTGAGTAC-3′ and the reverse primer was 5′-CGTTCAGCTCAGGGATGACC-3′ [16]. The presence of a single 412 bp band amplified with primers specific for GAPDH with the same cDNAs was used as an internal control under identical conditions.

bcl2 immunostaining. The 5 µm tumour sections were immunostained using the alkaline phosphatase–anti-alkaline phosphatase (APAAP) method [17] with the anti-bcl2 MAb (clone 124) [18] raised to a synthetic peptide. Briefly, paraffin sections were dewaxed in xylene and rehydrated through graded alcohols. The MAb bcl2 124, diluted 1:20, was applied overnight. A rabbit antimouse secondary antibody prediluted in 0.05 M Tris-buffered and containing normal swine serum was applied for 30 min. The alkaline phosphatase–mouse anti-alkaline phosphatase immune complex was first applied for 30 min and then for 10 min spaced by the use of the antimouse serum. The reaction was developed with alkaline phosphatase substrate containing naphthol AS-MX, Fast-red Tr and Levamisol (APAAP Kits, Dako). As a positive control for bcl2, we used a paraffin-embedded section from a normal peribronchial lymph node removed during post-surgical sampling of a lung tumour. At the same time, positive staining of small lymphocytes provided an internal control for bcl2 staining. Staining without the anti-bcl2 MAb was performed as a negative control. The count of bcl2 immunoreactivity was made by scoring a minimum of five high-power fields (40× objective lens).

p53 immunostaining. For p53 detection, the anti-p53 MAb PAb1801 (Oncogene Science, Manhasset, New York, U.S.A.) was used overnight at 1:200 dilution on 5 µm frozen sections, as previously reported [19]. This antibody reacts specifically with human wild-type and mutant p53 recognising the N-terminal epitope of the protein. The ABC method was used, developing the immunoreaction with diaminobenzidine. Simultaneous staining of a known p53 positive case was employed as a positive control for p53. Incubation of parallel slides omitting the first antibody was performed as a negative control. As for bcl2, the count of p53 immunoreactive cells was conducted by scoring a minimum of five high-power fields (40× objective lens). The median value of

this series (20% of positive cells) was used as the cut-off value to distinguish tumours with low p53 expression from tumours with high p53 expression.

Statistical analysis

All statistical analyses were carried out using STATISTICA software (Stat-soft). Univariate analysis was performed by modelling Kaplan–Meier survival curves. The Mantel–Cox test was used to evaluate the statistical significance of differences in survival distributions among prognostic groups. Multivariate analysis was carried out by using the Cox proportional-hazard model. Mann–Whitney and/or Kruskall–Wallis non-parametric tests were used to compare clinicopathological characteristics of the tumours with median immunostaining values. The a priori level of significance was set at a P value of less than 0.05.

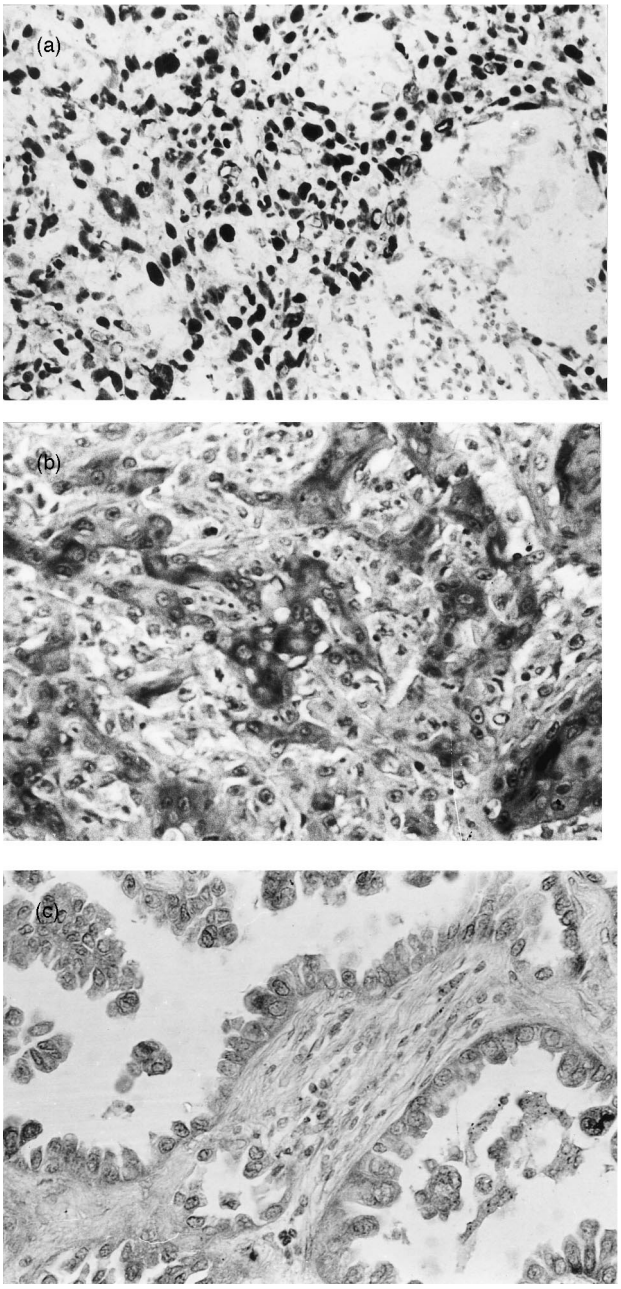

Figure 1. (a) p53 nuclear expression in a representative case of non-small cell lung carcinoma (avidin–biotin peroxidase complex (ABC) method, 25×). (b) bcl2 immunoreactivity in the cytoplasm of neoplastic cells (alkaline phosphatase–anti-alkaline phosphatase (APAAP) method, 25×). (c) Vascular endothelial growth factor (VEGF) expression in the cytoplasm of neoplastic cells (ABC method, 25×).

RESULTS

Clinico pathological characteristics

107 NSCLC patients (96 males and 11 females) were studied. There were 58 (54%) patients with squamous cell carcinoma, 43 (40%) patients with adenocarcinoma and 6 (6%) patients with large cell anaplastic carcinoma. 27 (25%) were classified as T1, 69 (64%) as T2, and 11 (10%) as T3. Metastatic involvement of hilar lymph nodes (N1) was present in 15 (14%), while mediastinal lymph nodes were affected in 22 cases (21%). No metastatic involvement was present in 70 cases (65%), 52 (49%) patients developed distant metastases during observation, and 69 (64%) were alive at the time of the analysis.

Gene protein expression and microvessel count (MVC)

p53 and bcl2 protein immunoreactivity was present in the nuclei (Figure 1a) and the cytoplasms of neoplastic cells (Figure 1b), respectively. Seventy-two of 107 tumours (67%) showed p53 immunoreactivity (mean 25.4 ± 28, range 0–90%; median 10%); bcl2 immunostaining was present in 58 of 107 cancers (54%) with a mean expression of 13.8 ± 22.6 (range 0–90%; median 3). bcl2 immunostaining was observed in some cases of normal bronchial epithelium, in particular in the basal layers of the bronchial walls. No p53 expression was detected in the surrounding non-neoplastic tissue.

A strong inverse association was found between bcl2 expression and MVC (Table 1). Sixty-four bcl2 negative tumours showed a significantly higher mean MVC (32.5 ± 17.9) compared with the bcl2 positive cancers (20.5 ± 14.3) ($P = 0.0004$). In contrast, a higher p53 nuclear immunoreactivity was related to a high mean MVC ($P = 0.03$) (Table 1). When analysing the relationship between bcl2 and MVC in the subgroups of p53 negative and p53 positive cancers, we found no significant correlations (data not shown).

Association of clinicopathological and biological parameters with survival

The prognostic impact of clinicopathological and biological parameters on patient survival was evaluated by univariate analysis. As shown in Table 2 and Figure 2, nodal status ($P < 0.000001$), MVC ($P < 0.000001$), bcl2 ($P = 0.002$), p53 ($P = 0.03$) and VEGF expression ($P < 0.000001$) significantly affected overall survival. Age, gender, histology and tumour size were unable to predict disease outcome. Multivariate

Table 1. *Association between bcl2, p53 and microvessel count (MVC) in* 107 *cases of non-small cell lung cancer*

Gene expression	Number of cases	MVC (mean ± S.D.)	P value*
bcl2 negative	64	3.25 ± 17.9	0.0004
bcl2 positive	43	20.5 ± 14.3	
p53 low	54	31.3 ± 18.3	0.03
p53 high	53	24.1 ± 16.1	

*Unpaired t test. S.D., standard deviation.

Table 2. Univariate analysis of the association between prognostic variables and overall survival in 107 cases of non-small cell lung cancer

Patient and tumour characteristics	Number of cases	Two-sided *P* value*
Sex		
Male	96	0.32
Female	11	
Age (years)		
≤ 64	64	0.95
> 64	45	
Tumour grade		
G1	22	0.57
G2	46	
G3	39	
Histology		
Squamous	58	0.24
Non-squamous	49	
Tumour size		
T1	27	
T2	69	0.19
T3	11	
Node status		
N0	70	
N1	15	< 0.000001
N2	22	
Microvessel count		
≤ 20	50	< 0.000001
> 20	57	
p53		
Low	54	
High	53	0.03
bcl2		
Negative	64	
Positive	43	0.002
VEGF		
Low	46	0
High	30	< 0.000001

*Cox–Mantel test. VEGF, vascular endothelial growth factor.

analysis, performed to define the variables with independent prognostic value with respect to survival, showed that MVC and VEGF expression alone retained their independent prognostic impact on overall survival ($P = 0.01$) (Table 3).

VEGF and gene protein expressions

The expression of VEGF was detected in 70 of 78 (90%) cases (mean 39.8, median 40, range 0–80%). VEGF protein was mainly present in the cytoplasm of neoplastic cells (Figure 1c), although some normal alveolar cells and some inflammatory cells showed VEGF immunoreactivity (data not shown). Vascular endothelium did not express the VEGF protein. VEGF protein expression was associated with p53 protein ($P = 0.04$) whereas an inverse association was found between bcl2 and VEGF immunoreactivity ($P = 0.007$) (Table 4). RT–PCR analysis revealed that VEGF mRNA was expressed in lung tumours of 23 of 26 patients examined (88%), confirming the data of VEGF protein expression. Three kinds of amplified cDNAs (VEGF 121, VEGF 165, VEGF 189) were detected in 23 (88%), 18 (69%) and 9 (35%) cases, respectively. Figure 3 shows the results of the electrophoretic analysis of PCR products in representative cases. In this small group of cases, we were unable to find any association between VEGF mRNA expression and p53 and bcl2 immunostaining (data not shown).

Table 3. Multivariate analysis of overall survival according to Cox's model

Variables	Beta	Standard error of beta	*t*	*P* value
Age	− 0.570	0.482	− 1.182	0.32
Gender	0.112	0.354	0.316	0.73
Tumour status	− 0.443	0.297	− 1.488	0.29
Node status	0.323	0.358	0.904	0.98
Histology	0.624	0.635	0.982	0.45
MVC	2.182	0.686	3.18	0.01
bcl2	− 0.276	0.577	− 0.478	0.73
p53	0.391	0.522	0.747	0.51
VEGF	1.959	0.606	3.23	0.01

MVC, microvessel count; VEGF, vascular endothelial growth factor.

Table 4. Association between bcl2, p53 and vascular endothelial growth factor (VEGF) expression in 107 cases of non-small cell lung cancer

	p53 expression			bcl2 expression		
Gene expression	Number of cases	Mean ± S.D.	*P* value*	Number of cases	Mean ± S.D.	*P* value*
VEGF low	46	19.9 ± 25	0.04	46	19.8 ± 24	0.007
VEGF high	31	32.6 ± 29		31	6.5 ± 13	

*Unpaired *t*-test. S.D., standard deviation.

DISCUSSION

The role of angiogenesis in the development and progression of human cancers has been widely studied [2], although a more complete knowledge of this phenomenon is obviously required.

Since, in NSCLC, angiogenesis seems to have an important role in metastasis, as widely reported both in early [7, 8, 20, 21] and late stage disease [22], we are interested in understanding the genetic regulation of the angiogenetic process. Several studies have highlighted the pivotal role of some growth factors in the promotion of tumour angiogenesis, in particular VEGF [23]. Moreover, VEGF, which seems to stimulate angiogenesis either through a proliferative or a chemoattractive mechanism on endothelial cells [24], has been recently related to tumour progression in different kinds of human cancers [25–27] including lung cancer [28, 29]. In NSCLC, which accounts for 70% of lung cancer, tumour progression and metastasis are considered to be related to alterations of some genes and/or tumour suppressor genes [30]. Moreover, tumour suppressor genes such as *p53* have been shown to influence angiogenesis by regulating the balance between stimulators and inhibitors of angiogenesis [10, 11] *in vitro*. In this study, we analysed the complex interrelationship between genes and suppressor gene protein expression and the angiogenetic pattern in a series of NSCLCs. Our results showed two interesting aspects: from a prognostic point of view, they confirmed both our recent and previous data concerning the association between MVC, bcl2, p53 and NSCLC behaviour [9, 12, 13]; from the point of view of neoangiogenesis regulation, they emphasised a

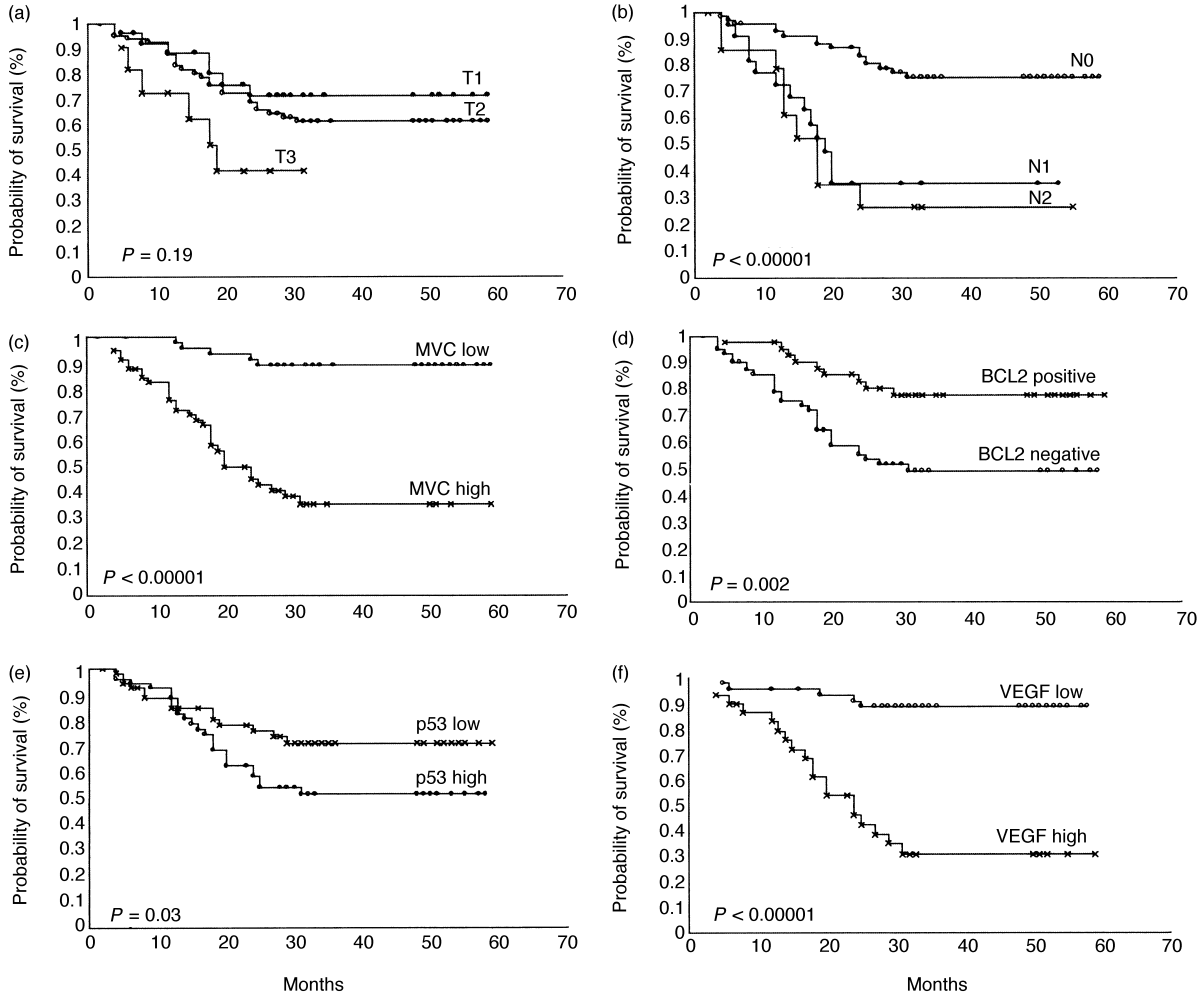

Figure 2. Survival curves of non-small cell lung cancer patients according to (a) size, (b) nodal status, (c) microvessel count, (d) bcl2, (e) p53 and (f) vascular endothelial growth factor expression (Kaplan–Meier method).

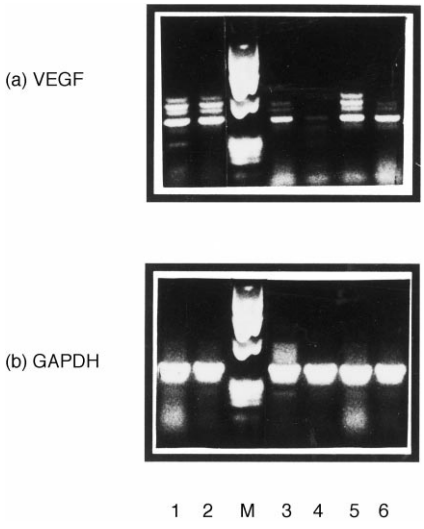

Figure 3. (a) Shows identification by reverse transcription–polymerase chain reaction (RT–PCR) of vascular endothelial growth factor (VEGF) isoforms expressed in human lung carcinomas. Expected VEGF amplification products of 452, 584, and 656 bp correspond to VEGF 121, VEGF 165, and VEGF 189, respectively. (b) Shows a single 412 bp band amplified with primers specific for GAPDH with the same cDNAs under identical conditions. Lanes 1–4: four non-small cell lung cancer samples.

strong relationship between bcl2 and p53 with MVC. Moreover, an interesting association was found between bcl2, p53 and VEGF. The influence of neoangiogenesis as well as VEGF expression have been related to relapse and poor prognosis in NSCLC, thus adding important information for the prognostic evaluation of this type of cancer [7–9, 20, 28, 29]. Knowing that the angiogenetic pattern may affect behaviour in lung cancer is very interesting from a clinical standpoint, since it could be very useful in selecting different postsurgical treatments. The development of new specific anti-angiogenic drugs for NSCLC could be introduced in the future.

The direct association between p53, MVC and VEGF has already been reported by our group [12] in a series of 105 NSCLCs, suggesting a putative influence of the *p53* tumour suppressor gene on VEGF expression. This has also been reported by Mukhopadhyay and associates [31] who demonstrated a suppressive effect of *VEGF* gene expression on an adenovirus transformed human fetal kidney cell line by wild-type p53. Moreover, Kieser and colleagues [11] showed that a mutant form of *p53* was involved in the 12-*O*-tetradecanoylphorbol-13-acetate induction of VEGF gene expression mediated by protein kinase C. The association between VEGF, MVC and p53 highlights the important role of VEGF in the control of neoangiogenesis in NSCLC and the hypothesis that wild-type p53 protein may stop cancer

development by attracting newly formed vessels. The inverse association in our study between bcl2 expression and MVC strongly agrees with their different impact on overall survival. The lack of association between VEGF mRNA expression, bcl2, p53 MVC could probably be related to the small number of cases analysed.

Several explanations exist for this inverse correlation. The bcl2 protein product could be involved in angiogenesis acting in different ways. Firstly bcl2 could downregulate p53, as demonstrated by *in vitro* analysis [32], so causing upregulation of thrombospondin-1 [10] and increasing angiogenesis. This possibility was not confirmed by our results, since we observed no inverse association between bcl2 and MVC in the subgroup of p53 positive tumours.

NSCLC cells expressing bcl2 could be protected from hypoxic alterations induced by factors such as tumour necrosis factor [33]: this could prevent necrosis and consequent hypoxic-induced VEGF upregulation. Low levels of VEGF might result in decreased neovascularisation of the tumours. This hypothesis is supported by the association found in our series between bcl2 and VEGF expression and could represent an exciting explanation for the role of the bcl2 protein in the acquisition of the angiogenic phenotype.

Further analyses, including transfection of lung cancer cells with bcl2 and/or wild-type p53 and the evaluation of VEGF levels in cultured cells, will be necessary to clarify the role of these genes in the regulation of angiogenesis.

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