

# Expression of 90K (Mac-2 BP) Correlates with Distant Metastasis and Predicts Survival in Stage I Non-Small Cell Lung Cancer Patients<sup>1</sup>

Antonio Marchetti, Nicola Tinari, Fiamma Buttitta, Antonio Chella, Carlo Alberto Angeletti, Rocco Sacco, Felice Mucilli, Axel Ullrich, and Stefano Iacobelli<sup>2</sup>

Departments of Oncology and Neurosciences, [A. M., N. T., F. B., S. I.] and Surgery [R. S., F. M.], University G. D'Annunzio, Chieti, Italy 66100; Department of Surgery, University of Pisa, 56124 Pisa, Italy [A. C., C. A. A.]; and Department of Biochemistry, Max-Planck Institute, 82152 Martinsried, Germany [A. U.]

## ABSTRACT

**90K**, also known as Mac-2 binding protein, is a secreted glycoprotein that binds galectins,  $\beta$ 1-integrins, collagens, and fibronectin, and has some relevance in cell-cell and cell-extracellular matrix adhesion. Previous studies have shown that serum levels of 90K have prognostic value in several neoplasms. In the present study, the role of the expression of 90K as an adverse prognostic indicator in 72 pathological stage I non-small cell lung cancer patients was investigated immunohistochemically. All of the patients underwent complete surgical removal of the tumor. The median length of follow-up care was 54 months. High level of 90K expression (90K staining of  $\geq 50\%$  of the neoplastic cells) was observed in 20 of the 72 (28%) tumors. Expression of 90K was confirmed by ELISA. The results showed that a high expression of 90K correlates with adverse prognosis. Among patients with high 90K expression, the disease-free and overall survival rates were significantly lower than the same rates of those with low expression ( $P = 0.0001$  and  $P = 0.0003$ , respectively). The incidence of distant metastases in the patients with high 90K expression (60%; 12 of 20 patients) was significantly higher than that of in the patients with low expression (21%; 11 of 53 patients;  $P = 0.0038$ ). The results of multivariate analysis confirmed that a high 90K expression was a significant factor to predict poor prognosis. We suggest that 90K expression could be a useful prognostic factor in patients with stage I non-small cell lung cancer, likely as an indicator of the metastatic propensity of the tumor.

## INTRODUCTION

Lung cancer is the leading cause of cancer-related death in the Western countries, with NSCLC<sup>3</sup> accounting for  $\sim 80\%$  of the cases. The anatomical extent of cancer as reflected by the tissue-node-metastasis classification has traditionally been the most important determinant of prognosis in NSCLC. However, as it is the case with so many malignancies, the tissue-node-metastasis-based categories have proven to be often inadequate in predicting the prognosis of individual patients. In fact, patients with NSCLCs in comparable stages may have different clinical courses. This is true even for patients having stage I NSCLC;  $\sim 30\%$  of them still die after complete surgical resection (1). This is related to the fact that occult metastases may be present at the time of surgery. The identification of new biological markers able to predict the risk for a patient of relapse after resection of stage I NSCLC would facilitate the development of postresection treatment strategies to be used for high-risk patients. Many possible biological markers have been reported, such as Ki-67 (2), c-erbB-2 (3), p53 and ras p21 (4, 5), cyclins (6, 7), and polysialic acid (8), but none of them have gained routine clinical use in the postsurgical evaluation of these patients.

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<sup>2</sup>To whom requests for reprints should be addressed, at Cattedra di Oncologia Medica, Palazzina Scuole di Spec., Università G. D'Annunzio, Via dei Vestini, 5, 66100 Chieti, Italy. E-mail: iacobelli@unich.it.

<sup>3</sup>The abbreviations used are: NSCLC, non-small cell lung cancer; Mac-2BP, Mac-2 binding protein; IL, interleukin.

A large oligomeric protein composed of  $\sim 90$ K subunits, designated 90K/Mac-2BP, has been originally identified as a tumor-associated antigen (9, 10) and as a ligand of galectin-3 (formerly Mac-2; Refs. 11, 12). Characterization of 90K by cDNA cloning and sequencing revealed a multidomain organization of the 567 amino acid residues mature protein. Notably, a region in the NH<sub>2</sub>-terminal portion of the protein shows a high degree of homology with members of the macrophage scavenger receptor cysteine-rich domain superfamily (12, 13). The functions of 90K are not well defined yet, but it has been suggested that the protein, as other members of its family, is involved in the host response to tumors and infections (13), and this is consistent with the observation of a down-modulation of the proinflammatory response in 90K knockout mice (14). More recently, several reports pointed out a role of the protein in the cell adhesive processes. In particular, it has been shown that 90K, as a ligand of galectin-3 and galectin-1, may promote homotypic cell-cell contacts (15, 16) or regulate cell adhesion (17). Moreover, the protein was shown to bind collagens and fibronectin, thus to be located in the extracellular matrix, and to promote cell adhesion and spreading via its binding to  $\beta$ 1-integrins (18, 19).

90K is present in the  $\mu\text{g/ml}$  range in serum and other biological fluids (9, 12) of the normal subjects, where it may exert some of its functions. Its serum levels are not influenced by age, gender, or smoking habit (10). Elevated levels of 90K are often observed in patients with different types of cancer and have proven to be of prognostic value. For example, serum levels  $\geq 11 \mu\text{g/ml}$  were associated with a poor survival and metastatic spread to liver in a series of 375 patients with operable breast cancer (20). However, there is no information about 90K expression in lung cancer and its potential clinical application in this tumor type.

In the present study, we examined by immunohistochemistry the expression pattern of 90K in a monoinstitutional series of 72 patients with completely resected stage I NSCLC to assess its prognostic importance. We found that the protein was overexpressed in  $\sim 30\%$  of the tumors analyzed and that this overexpression was associated with a significantly worse outcome for patients with stage I NSCLC.

## MATERIALS AND METHODS

**Study Population.** The primary NSCLC specimens were archived tissue samples of surgically resected pathological stage I tumors from 72 consecutive patients treated at the Department of Surgery, University of Pisa (Pisa, Italy) between 1992 and 1993. Patient stage was determined according to the guidelines of the American Joint Committee on Cancer (21). The pathological analyses were done in a blinded manner in regard to the clinical information of the patients. All of the patients were treated by surgery alone and received a median of 54 months of follow-up (range, 7–94 months) after surgical treatment. Follow-up was conducted with intervals of 3 months for the first year and 6 months thereafter. Recurrences were detected by imaging techniques and confirmed, when required, by histological examination of bioptic material. At completion of follow-up, patients were categorized as alive with evidence of disease, alive without disease, and dead as a result of lung carcinoma. No patient in this series had died of cancer-unrelated cause. The study population consisted of 67 (93%) men and 5 (7%) women, with a mean age of 63.6 years (range 43–75 years). Histological type and tumor cell differentiation were

determined according to the WHO criteria (22). The specimens included 36 (50%) squamous cell carcinomas, 25 (35%) adenocarcinomas, 6 (8%) bronchioloalveolar carcinomas, and 5 (7%) anaplastic large cell carcinoma. Twenty (28%) tumors were well differentiated (G1), 28 (39%) moderately differentiated (G2), and 24 (33%) poorly differentiated (G3).

**Immunohistochemistry.** Formalin-fixed, paraffin-embedded, 5- $\mu$ m tissue sections from all 72 of the primary tumors were stained for the 90K protein using the mouse monoclonal antibody 1A4.22 (23). Sections were mounted on polylysine-coated slides and deparaffinized with two changes of xylene. Sections were then treated with 0.3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidase activity, microwaved in citrate-phosphate buffer [0.05 M citric acid, 0.1 M sodium phosphate dibasic (pH 6)] for antigen retrieval, and incubated with 10% normal swine serum for 30 min to block nonspecific binding. The anti-90K monoclonal antibody was then applied at the concentration of 2  $\mu$ g/ml for 30 min at room temperature followed by a standard staining procedure using the LSAB (labeled streptavidin-biotin) peroxidase kit (Dako, Glostrup, Denmark). The slides were then rinsed in tap water, counterstained with 5% hematoxylin, dehydrated, cleared in xylene, and mounted in permanent coverslipping medium. Positive controls were two known positive cases of human lung cancer. Negative controls were obtained by replacement of primary antibody with buffer.

All of the immunostained sections were examined by three pathologists in a blinded fashion. In case of discordance (four cases) the tissue was retested. In all of these cases, concordance was reached. Positive cells were counted under a microscope  $\times 400$  field ( $\times 40$  objective and  $\times 10$  ocular lens; 0.180 mm<sup>2</sup>/field). Quantitation of the percentage of cells expressing 90K was then performed in a two-grade system: low and high expression for 90K were defined as  $<50\%$  and  $\geq 50\%$  of the neoplastic cells being immunostained, respectively. The percentages of tumors having  $<25\%$  or  $>75\%$  of 90K positive cells were 49% and 7%, respectively.

**ELISA for 90K.** Frozen tissue specimens from corresponding tumor material were disrupted into small pieces and homogenized with 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 0.4 mM Pefabloc (Boehringer, Mannheim, Germany). Homogenates were centrifuged at 14,000  $\times g$  for 15 min at 4°C to obtain the supernatant fraction (cytosol). Cytosolic 90K levels were determined with an ELISA kit (Diesse, Siena, Italy) according to the manufacturer's instructions. Results were expressed in  $\mu$ g 90K/mg protein.

**Statistical Methods.** The relationships between 90K expression and clinicopathological parameters were assessed by Fisher's exact test or  $\chi^2$  test as appropriate. The survival curves were estimated using the Kaplan-Meier

method and differences among them evaluated by the log-rank test. Disease-free survival was defined as the period between surgery and the first local recurrence, the evidence of distant metastasis or the end of the study. Overall survival was defined as the period from surgery to the death of the patient. Cox's proportional hazards regression model was used to assess the impact of 90K expression on disease-free and overall survival after adjustment for tumor size (T<sub>1</sub> versus T<sub>2</sub>), histological type (SCC versus other histotypes), and tumor grade (G1–2 versus G3). The assumption of the proportional hazards model was checked by plotting the log of the cumulative hazard function. A  $P < 0.05$  was considered as significant.

## RESULTS

**90K Expression in NSCLC.** 90K expression was found to be high (staining of  $\geq 50\%$  of the neoplastic cells) in 20 of 72 (28%) tumor specimens. Immunohistochemical staining was either homogeneous or with a heterogeneous pattern, which was present over the whole tumor section; focal staining was rarely observed. The staining was cytoplasmic and granular in all instances. The cytoplasmic staining was diffuse in squamous cell carcinomas and apical in adenocarcinomas characterized by tall columnar cells. A weak apical staining was also evident in columnar cells of the normal bronchial epithelium. Examples of 90K immunostaining in different histotypes are shown in Fig. 1. No statistically significant association was found between 90K expression and other clinicopathological variables such as age, sex, histological type, tumor size, and degree of tumor differentiation (Table 1).

To verify whether the quantitative assayment of the protein levels correlated with its immunohistochemical expression, an ELISA of 90K was performed on the cytosols of 14 randomly selected tumors. These samples showed different levels of protein expression in immunohistochemistry. We found a strong association between immunohistochemical evaluation of 90K expression and the levels of the protein determined by ELISA (Fig. 2). The mean 90K content in ELISA was  $0.39 \pm 0.31$   $\mu$ g/ml ( $n = 10$ ) for tumors with low immunohistochemical expression and  $1.21 \pm 0.87$   $\mu$ g/ml ( $n = 4$ ) for those with high expression. The differences in expression were statistically significant ( $P = 0.017$ ).

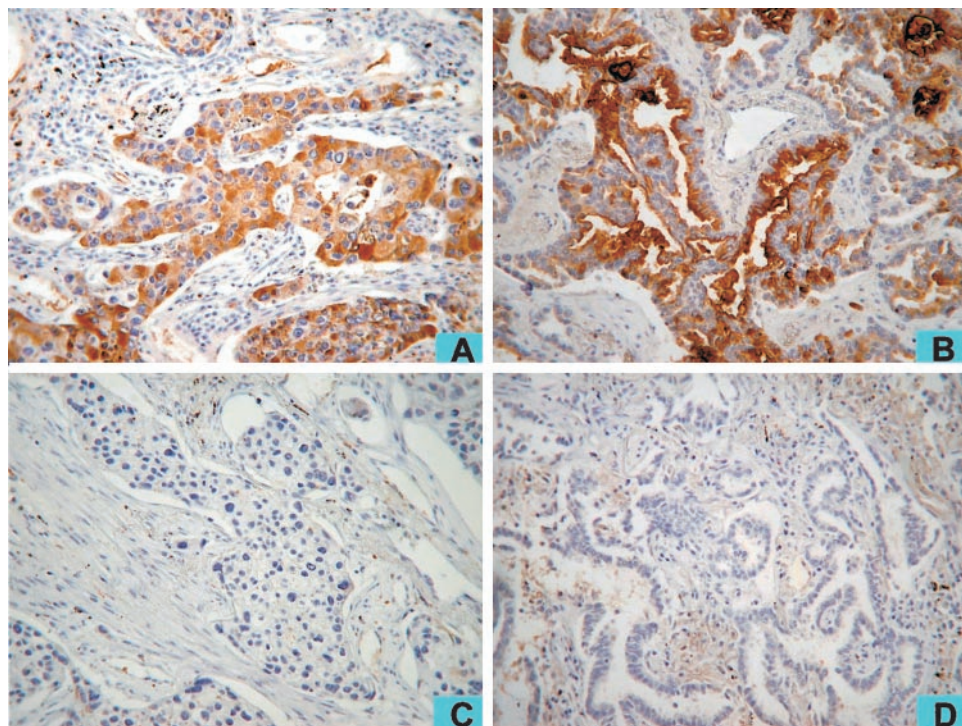


Fig. 1. Immunohistochemical staining of NSCLC tissues with anti-90K 1A4.22 antibody. A strong cytoplasmic reactivity is evident in a squamous cell carcinoma (A) and in an adenocarcinoma (B). Negative immunostaining of a squamous cell carcinoma (C) and an adenocarcinoma (D).

Table 1 90K status in stage I NSCLC tumors according to the clinicopathological features of patients

Variable	90K low n (%)	90K high n (%)	P
Age (mean $\pm$ SD, yr)	63.8 $\pm$ 7.1	63.2 $\pm$ 6.3	0.72
Sex			
Male	50 (96)	17 (85)	0.13
Female	2 (4)	3 (15)	
Tumor differentiation			
Well (G1)	14 (27)	6 (30)	0.92
Moderate (G2)	20 (38)	8 (40)	
Poor (G3)	18 (35)	6 (30)	
Histological type			
SCC <sup>a</sup>	25 (48)	11 (55)	0.90
AD	19 (36)	6 (30)	
BAC	4 (8)	2 (10)	
LCC	4 (8)	1 (5)	
Tumor size			
T1	12 (23)	7 (35)	0.37
T2	40 (77)	13 (65)	
Distant metastases			
Present	11 (21)	12 (60)	0.0038
Absent	41 (79)	8 (40)	

<sup>a</sup> SCC, squamous cell carcinoma; AD, adenocarcinoma; BAC, bronchioloalveolar carcinoma; and LCC, large cell carcinoma.

**90K Expression and Distant Metastasis.** In our study population, 23 (32%) patients developed distant metastasis at various time intervals from complete resection, and this resulted to be strongly associated with the level of 90K expression in the primary tumor. Although 21% (11 of 52) of patients with low 90K expression developed distant metastasis, this percentage raised to 60% (12 of 20) among patients with high 90K expression ( $P = 0.0038$ ).

**90K Expression and Postoperative Survival.** The Kaplan-Meier postoperative survival curves showed a significantly worse prognosis for patients with high 90K expression. The 5-year disease-free survival rates for high-90K and low-90K patients were 31% and 78% ( $P = 0.0001$ ), respectively. 90K expression proved to affect overall survival as well; the 5-year survival rates were 36% for the 20 patients with high 90K expression and 80% for the 52 patients with low 90K expression ( $P = 0.0003$ ; Fig. 3).

There was no significant correlation between disease-free or overall survival and tumor size, histological type, and degree of tumor differentiation. The multivariate analysis confirmed that 90K expression was the only independent and significant factor to predict poor prognosis for both disease-free ( $P = 0.0003$ ) and overall survival ( $P = 0.0005$ ; Table 2).

## DISCUSSION

In the present study, on a population of 72 patients with pathological stage I NSCLC we evaluated by immunohistochemistry the expression of the protein 90K/Mac-2BP, whose serum levels in the past have been reported to have prognostic values in several neoplasms, such as breast carcinoma (20), ovarian carcinoma (24), and lymphoma (25).

Here, we showed that 90K is overexpressed in a large fraction of pathological stage I NSCLCs, with 20 of 72 samples (28%) showing a positivity of  $>50\%$  of the cells. When the immunohistochemical patterns of expression were analyzed comparatively with the follow-up data, they clearly stratified the patients into markedly different survival groups; patients with high 90K expression in the tumor had a significantly shorter overall and disease-free survival times than those with low 90K expression. In our judgement, the fact that all of the patients were treated at a single institution and received a long follow-up after surgery makes survival analysis quite reliable. Moreover, the expression of 90K resulted to be significantly associated with the establishment of distant metastasis. No correlation was found

between the expression of 90K and tumor size, histological type, or degree of tumor differentiation, thus supporting the contention that the prognostic significance of 90K expression is different from that of these conventional prognostic indicators.

It is likely that 90K prognostic value results from its correlation with the occurrence of distant metastasis. A positive association between high serum levels of 90K and development of metastasis during the postsurgery follow-up has been already reported for node-positive breast cancer patients (20). How 90K overexpression intervenes in tumor metastasis remains to be established. However, we believe that some of the functions that have been described for this protein may shed light on its involvement. The journey of the cancer cells in the bloodstream is a recognized limiting step in metastatization and is largely responsible for its inefficiency; only 1 cell in 10,000 survives the mechanical stresses of

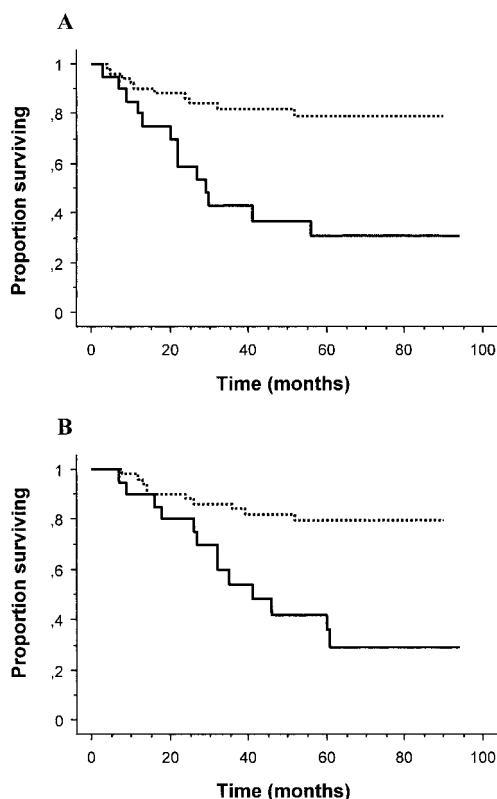


Fig. 2. Disease-free (A) and overall survival (B) in the 72 NSCLC patients based on high or low 90K expression

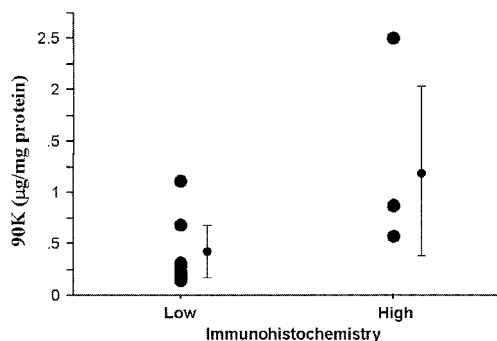


Fig. 3. Association between 90K expression evaluated immunohistochemically and protein levels determined by ELISA. Fourteen randomly selected tumors were analyzed by immunohistochemistry and regarded as high or low. The same tumors were assayed for 90K levels by ELISA. The graph shows the quantitative 90K levels of high and low samples. Also indicated are means; bars,  $\pm$ SD. The difference was statistically significant ( $P = 0.017$ ).

Table 2 Multivariate analysis of prognostic variables for disease-free and overall survival

Variable	Disease-free survival				Overall survival			
	$\beta$	SE	HR (95% CI)	P	$\beta$	SE	HR (95% CI)	P
Tumor differentiation G1–G2/G3	0.80	0.52	0.45 (1.23–0.16)	0.12	0.78	0.51	0.46 (1.25–0.17)	0.13
Histological type SCC <sup>a</sup> /non-SCC	0.42	0.42	0.66 (1.50–0.29)	0.32	0.36	0.42	0.70 (1.58–0.31)	0.32
Tumor size T <sub>1</sub> /T <sub>2</sub>	0.63	0.51	0.53 (1.46–0.20)	0.22	0.66	0.51	0.52 (1.41–0.19)	0.20
90K expression Low/high	1.70	0.43	5.50 (12.87–2.34)	0.0003	1.62	0.43	5.04 (11.72–2.16)	0.0005

<sup>a</sup> SE, standard error; HR, Hodds ratio; CI, confidence interval; SCC, squamous cell carcinoma.

the circulation and eventually homes in a new tissue (26). It has been demonstrated that, through its binding to galectin-1 and -3, 90K mediates homotypic adhesion of melanoma cells and the formation of multicellular aggregates (15), thus giving a positive contribution to one of the mechanisms thought to improve the survival rate of blood-borne cancer cells. Another key step in metastatic diffusion is the adhesion of cancer cells to endothelia, which prelude to their escape from vasculature, and is regulated by mechanisms similar to those used by leukocytes. The interaction between tumor and endothelial cells is known to involve molecules produced by both cell types and by other cells within the vascular compartment. It has been shown that in tumor implants overexpressing 90K, endothelial cells are strongly positive for VCAM-1 and ICAM-1, in contrast to controls (27). It is not known whether the induction of these adhesion molecules is mediated directly by 90K or through secretion of IL-1, IL-6, and tumor necrosis factor  $\alpha$  by local peripheral blood mononuclear cell exposed to tumor-released 90K (28). In this respect, 90K may act as the carcinoembryonic antigen produced by colon cancer cells, which has been shown to induce ICAM-1 in endothelial cells by promoting the release of the very same cytokines (IL-1, IL-6, and tumor necrosis factor  $\alpha$ ) by hepatic sinusoidal Kupffer cells (29). Finally, 90K has been found to be deposited in the extracellular matrix where it interacts with collagens and fibronectin (18), and to mediate cell adhesion via binding to integrins (18, 19).

According to the above considerations, it seems likely that, after detaching from the primary tumor, cancer cells expressing high levels of 90K may locally release the protein at high concentrations and create microenvironments favoring their survival in the bloodstream (through induction of cell aggregation), their adhesion to endothelia (through induction of VCAM and ICAM), and to extracellular matrix, hence the establishment of new tumoral colonies.

Several prognostic indicators have been reported for stage I NSCLC (2–8, 30, 31), and some of them, such as intensity of angiogenesis (32) and secretion of soluble IL-2 receptor (33) are considered to be markers of metastatic propensity. However, in other settings they failed to predict survival among patients undergoing resection for NSCLC (34). We believe, as others noted (30), that to fully appreciate the prognostic value of the different indicators it is mandatory to evaluate the interrelationships among them with the aim to develop a comprehensive prognostic index that integrates the different reported biomarkers. Several reports published recently support this belief (35–37). The results of the present study demonstrate that the protein known as 90K can be regarded as one of the biomarkers worth inclusion in the development of a prognostic model for stage I NSCLC, likely as an indicator of the metastatic propensity of the tumor.

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