Prognostic index expression of cyclin-D1, cerbB-2 and VEGF: metastases *vs* corresponding primary cancers and metastatic *vs* non-metastatic adenocarcinomas

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Summary. The prognostic relevance of different molecular markers in lung cancer is a crucial issue still worth investigating, and the specimens collected and analyzed represent a valuable source of material. Cyclin-D1, c-erbB-2 and vascular endothelial growth factor (VEGF) have shown to be promising as prognosticators in human cancer. In this study, we sought to examine the importance of Cyclin-D1, c-erbB-2 and VEGF, and to study the quantitative relationship among these factors and disease progression in metastases vs corresponding primary cancer, and metastatic vs non metastatic cancers. Material and Methods: We used immunohistochemistry and morphometric analysis to evaluate the amount of tumour staining for Cyclin-D1, c-erbB-2 and VEGF in 52 patients with surgically excised ademocarcinoma of the lung, and the outcome for our study was survival time until death from hematogenic metastases. Results: Metastasis presented lower c-erbB-2 expression than corresponding primary cancers (p=0.02). Cyclin-D1 and VEGF expression were also lower in metastases than in corresponding primary cancers, but this difference did not achieve statistical significance. Non-metastatic cancers also presented significantly lower Cyclin-D1 and c-erbB-2 expression than metastatic cancers (p<0.01 and p<0.01, respectively). Equally significant was the difference between higher c-erbB-2 expression by metastatic cancers compared to non-metastatic cancers (p=0.02). Considering survival in Kaplan-Maier analysis, Cyclin-D1 (p=0.04), c-erbB-2 (p=0.04) and VEGF (p<0.01) were important predictors of survival in metastatic cancers.

Conclusion: Different tumour cell profiles in metastases, corresponding primary cancers, and non-metastatic cancers were found, thus suggesting that different cell clones control the invasive and non-invasive behaviour of the cancers. The fact that cancers with higher indexes of Cyclin-D1, cerbB-2 and VEGF expression have the capacity to metastasize offers us the potential to guide the use of adjuvant chemotherapy in patients likely to fail treatment after surgical excision of cancers.

Key words: Cyclin-D1, c-erbB-2, VEGF, lung cancer, prognostic

Introduction

The incidence of lung cancer continues to rise despite a slight decline, particularly in men, for the first time in decades. It is now the leading cause of cancer deaths in both sexes in Europe, United States and Brazil. (Younes, 1997; Janssen-Heijnen et al., 1998; Wingo et al., 1998). The overall 5-year survival for cases of non-small cell lung cancer (NSCLC) is only 10 to 15%, largely because the minority of cases present with limited stage of disease that is amenable to curative surgical resection. Despite advances in surgery, chemotherapy, and radiotherapy in the last two decades, the death rate has changed very little. Subjects can have markedly different rates of disease progression in NSCLC4, some with metastases and others with limited disease, suggesting that these tumours, despite having the same histological subtype, are biologically different. Moreover, treatment success is highly dependent on proper patient selection, illustrating the need to identify different cell profiles in tumours that may help clinicians to optimise therapeutic efforts for each patient,

individually.

In this regard, many have studied molecular or other types of markers in the primary tumour to discover what might be related to advanced disease and shortened survival (Baattlehner et al., 1993; Capelozzi et al., 1993; Bernardi et al., 1995, 1997; Antonangelo et al., 1997; Carvalho et al., 1997, 2000; Rodrigues et al., 1997; Caporrino et al., 1999; Demarchi et al., 2000) . Because cell cycle alteration and growth factor deregulation have been thought to be important in tumour invasion and metastases, a group of factors have been targeted as potentially useful tumour markers[15,16].

Among these, Cyclin-D1, c-erbB-2 and VEFG have shown to be promising. Cyclins are key components of cell cycle progression machinery. They activate their partners, cyclin-dependent kinases (CDKs), which sequentially phosphorylate critical substrates that regulate the progression of the cell cycle. Cyclin-D1 is expressed early in the G1phase and degrades prior to the entrance into phase S (Jiménez-Orozco, 2001; Petty et al., 2003; Dragnev et al., 2003). Molecular alterations of Cyclin-D1 occur early in lung carcinogenesis and Cyclin-D1 overexpression can be immunodetected. When Cyclin is overexpressed, the cell decreases transition through the cell cycle (G1-S-G2-M) and perhaps limits the potential for repair of DNA damage caused by carcinogen exposure.

c-erbB-2 (HER-2/neu) is a proto-oncogene located in the long arm of chromosome 17. It codifies for a 185-kd protein that belongs to the epidermal growth factor receptor family and displays tyrosine kinase activity. Although HER-2/neu has been studied most extensively in breast cancer and its expression is limited to cancer cells, relatively little information is available regarding its prognostic value in lung cancer progression (Han et al., 2002).

Vascular endothelial growth factor (VEGF) is a 45 kDA glycoprotein, homodimeric, basic, and able to bind heparin. It is a major regulator for angiogenesis, and binds and activates two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1). These receptors regulate physiological, as well as pathological angiogenesis. VEGFR2 has strong tyrosine kinase activity, and transduces the major signals for angiogenesis21. Mutagenesis studies have shown that only a small number of VEGF residues are important and essential for the binding with RTK. Data described to date from the studies of VEGF/RTK interactions agree with the hypothesis that KDR receptor is the main human receptor responsible for VEGF activity in both physiological and pathological vascular development 22.

In some studies, immunostaining for Cyclin-D1, c-erbB-2 and VEGF has also been found to be significantly associated with the early stages of lung cancer (Nishio et al., 1997; Keum et al., 1999; Gugger et al., 2001), but its expression in advanced disease stages is still to be clarified. Aiming at analysing the importance of Cyclin-D1, c-erbB-2 and VEGF and exploring the quantitative relationship among these factors and progression of disease, we compared these markers in metastases vs corresponding primary adenocarcinomas and metastatic vs non-metastatic adenocarcinomas and herein report our results.

Materials and methods

Patients and tumor tissues

During 1992 to 2003, from all patients with a diagnosis of primary lung adenocarcinoma who received surgical treatment at the Thoracic Surgery Department of Hospital das Clinicas of the University of São Paulo Medical School, AC Camargo Hospital of Cancer, Hospital das Clinicas of Marilia Medical School and Mogi das Cruzes School of Medicine (São Paulo, Brazil), 52 patients (36 males and 16 females), mean age 63 (range, 38 to 90 years) were selected for this study. Among these 52 patients, 27 were also submitted to surgical resection or biopsies of hematogenic metastasis, 6 months after the primary tumor resection. Only patients with a confirmed postoperative diagnosis were selected for the study, based on the histopathological diagnosis, and availability of paraffin-embedded blocks of the primary tumours and their metastases.The preoperative staging studies included routine history and physical examination, biochemical survey, chest X-ray, bronchoscopy with biopsies, CT scan of thorax and upper abdomen, abdominal ultra sound, and bone scan; preoperative CT scan of the brain was routinely performed. As the main purpose of this study was to study the profile of primary lung adenocarcinoma in terms of invasiveness and metastatic potential, final pathological staging was considered for the analysis. The tumour stages were classified according to the international union against cancer's Tumour-Node-Metastases classification (Montain, 2000; Gugger et al., 2001). Among the 62 patients with primary lung adenocarcinoma, nine patients were in stage I, 8 in stage II, 8 in stage III and 27 in stage IV. Follow-up studies included physical examination, chest X-ray, and blood tests at 3-month intervals, and an additional thoracic CT scan, abdominal ultrasound, and bronchoscopy if necessary. Close follow-up was documented and updated until 2006 by contacting family practitioners through questionnaires concerning local relapse, distant metastases, and death.

Primary cancer tissue samples were obtained during first surgical resection (first operation) and matching metastases samples were obtained by diagnostic procedures or biopsies during the course of the disease (second operation), and fixed in 10% formalin. Their respective paraffin-embedded blocks were sectioned at 3 µm-thickness and stained with Haematoxylin and Eosin (H&E) (Fig. 1A,B). The histological classification of each case was assessed by light microscopy according to the World Health Organization guidelines (Travis et al., 1999).

Following the initial diagnostic evaluation, the 52

patients with lung adenocarcinoma were divided into the following groups: 1) metastases and corresponding primary cancers (N=27), 2) non-metastatic cancers (N=25).

Immunohistochemistry

The presence of cyclin-D1, c-erbB-2 and VEGF were analysed by immunohistochemical staining using a LSAB kit, (Dako Co, Carpinteria, CA, K0690) in a 3 mm-thick section and mounted onto Silane-coated slides (Sigma – cod: A3648). The antibodies used were monoclonal mouse antihuman Cyclin-D1 (Clone DCS6, Dako A/S, Glostrup, Denmark, M7155, at a dilution of 1:320); c-erbB-2 rabbit anti-human antibody (Dako A/S, Glostrup, Denmark, A0485, at a dilution of 1:50); and VEGF (clone A20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; dilution, 1:800). Brownish nuclear staining was considered evidence of the Cyclin-D1 antigen expression, whereas brownish cytoplasmic staining was evidence for the c-erbB-2 and VEGF antigen expression by cells

Morphometry

The immunostaining intensity and distribution were evaluated by hot spots of the periphery of the tumor at the peripheral edge of the proliferation, and then quantified by conventional point-counting technique (Gundesen et al., 1988). Briefly, in this method, a standard microscope equipped with an eyepiece (x10) containing a reticulated grid with 100 points and 50 lines was used for measurements. At a magnification of x400, the hot spots in each case were studied to quantify the amount of points overlying stained cells. The density of stained positive cells in hot spot areas of tumour tissue represents the immunostaining indexes of expression and were obtained by the following correlation:

$$IIE = Pp / Pt = (\% \text{ section})$$

Where Pp / Pt represents the numbers of points overlying stained tumour cells and total tissue or 1,000 points, respectively, expressed as a percentage.

Statistical analysis

Mean and range values for each parameter evaluated were plotted in tables. Before proceeding to a formal analysis of data, descriptive statistics were recorded and graphically illustrated. To verify differences in variables from metastases *vs* corresponding primary cancers and metastatic vs non-metastatic cancers, we proceeded toward the formal analysis of the data. In this context, the first analysis assessed differences and associations in the frequency of variables in metastases vs corresponding primary cancer. For this reason, the T-test was applied using paired sample statistics, paired sample correlation, and paired sample tests. The second analysis assessed differences and associations in the frequency of variables in non-metastatic cancers *versus* metastatic cancers using the T-test and ANOVA, with Tukey-HSD or Dunnett-T3 post-hoc test for multiple comparisons, after Shapiro-Wilk's tests for normality and Levene's one-way analysis for homogeneity of variance. Furthermore, comparisons of markers with survival were made by Kaplan-Meier curves. All statistical procedures were performed with SPSS version 10.0 statistical software (SPSS, Inc., Chicago, IL). The level of significance was set at p= 0.05.

Results

Table 1 summarizes patient's characteristics. For survival analyses, the median follow-up duration was 60 months (range, 1-121 months). Relapse was defined as diagnosis of distant metastases or local recurrence. Within the observation period, a total of 33 patients (63%) of the 52 eligible patients died of cancer-related causes. Throughout the course of the initial evaluation, 27 (51%) of these patients were at the M1 stage. In this subgroup hematogenic metastases were local in 4 patients (14%), brain in 8 patients (29%), bone in 8 patients (29%), liver in 2 patients (7%), soft tissue in 4 patients (14%), and adrenal in 1 patient (1%).

Table 2 summarizes the morphometric results found in metastases, corresponding primary cancers and non-metastatic cancers.

Histological variables were evaluated and compared in 27 metastases and their corresponding primary cancers. Cyclin-D1 was negative in 8/27 (29%)

Table 1. Patient's characteristics.

Total no. of patients	52
Age (yrs)	63 (38-90)[a]
Gender	36 males, 16 females
Overall stage	
I	9
II	12
III	8
IV	33
T stage	
1	24
2	15
3	7
4	1
N stage	
0	25
1	19
2	5
3	3
Follow-up (months)	39 (1-133)[a]
Patients censored for survival analysis at last time of follow-up	33

[a]: values represent median (range).
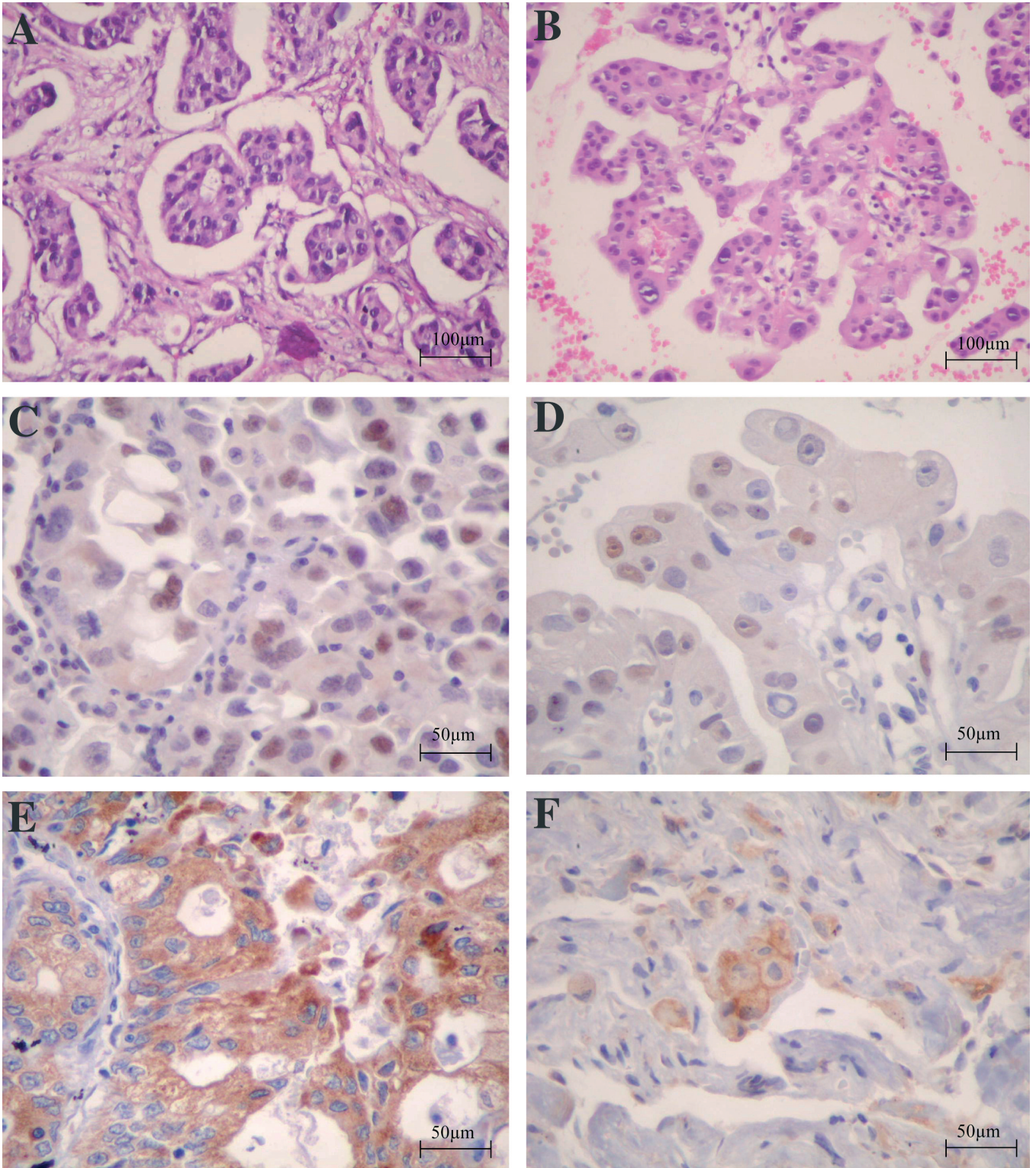
Fig. 1. Primary lung adenocarcinoma **(A)** and metastatic lung adenocarcinoma **(B)** seen at H&E staining. x 200. Immunohistochemical staining for Cyclin-D1 demonstrating lower expresion in cell nuclei seen in primary tumour **(C)** compared to metastatic tumour **(D)**. Immunohistochemical staining for c-erbB-2 demonstrating minimal staining of primary tumour cell cytoplasm **(E)** compared to metastatic lesion **(F)** and equally immunohistochemical staining expression for VEGF in primary tumour **(G)** when compared to metastatic tumour (H). x 400

Table 2. Summary of morphometric results[a].

Sub-Groups and Histological Variables	Mean±SEM
Primary metastatic tumor (N=27)	
Cyclin D1 (% of points)	44.70±6.58
c-erbB-2 (% of points)	48.88±4.29
VEGF (% of points)	31.12±7.05
Hematogenic metastasis (N=27)	
Cyclin D1 (% of points)	10.22±3.33
c-erbB-2 (% of points)	30.92± 4.47
VEGF (% of points)	13.70±4.76
Primary non metastatic tumor (N=25)	
Cyclin D1 (% of points)	8.44±2.92
c-erbB-2 (% of points)	13.60±2.74
VEGF (% of points)	20.95±5.83

[a]: The units of "of points" indicate the number of points overlying the phenomena of interest divided by the total number of points overlying tumor. In morphometry, this is called a point fraction and is often symbolized as Pp. Pp has been shown to approximate the volume fraction or Vv (refer 25).

metastases and 6/27 (22%) corresponding primary cancers. c-erbB-2 expression was negative in 3/27 (11%) metastases and positive in all 33 corresponding primary cancers (100%) and VEFG expression was negative in 13/27 (48%) metastases and positive in 11/27 (40%) corresponding primary cancers. Metastasis presented lower c-erbB-2 expression than corresponding primary cancers (13.60±2.74, p=0.02 vs 30.92±4.47.). Cyclin-D1 and VEGF expression were lower in metastases than in corresponding primary cancer, but this difference did not achieve statistical significance.

Histological variables were also evaluated in 25 non-metastatic cancers and then compared to 27 metastatic cancers. Cyclin-D1 was negative in 11/25 non-metastatic cancers (44%), c-erbB-2 was negative in 6/25 (24%) and VEGF was negative in 7/25 (28%). Non-metastatic cancers presented significantly lower cyclin D1 and c-erbB-2 expression than metastatic cancers (8.44±2.92 vs. 44.70±6.58%, p<0.01) (Fig. 1C,D) and (13.60±2.24 vs. 48.88±4.29%, p<0.01), respectively (Fig. 1E,F). Equally significant was the difference between higher c-erbB-2 expression by metastatic cancers compared to non-metastatic cancers (20.6% vs 17.6%, p=0.02) (Fig. 1G,H).

Considering survival in Kaplan-Maier analysis, the most important predictors of survival in metastatic cancers were Cyclin D (p=0.04), c-erbB-2 (p=0.04) and VEGF (p<0.01) expression (Fig. 2).

Discussion

Adenocarcinomas of the lung frequently metastasize to distant organs. Although the metastatic potential of lung adenocarcinomas is lower than that of small cell carcinomas, distant spread is demonstrable at diagnosis presentation in over one-third of the cases. The usual
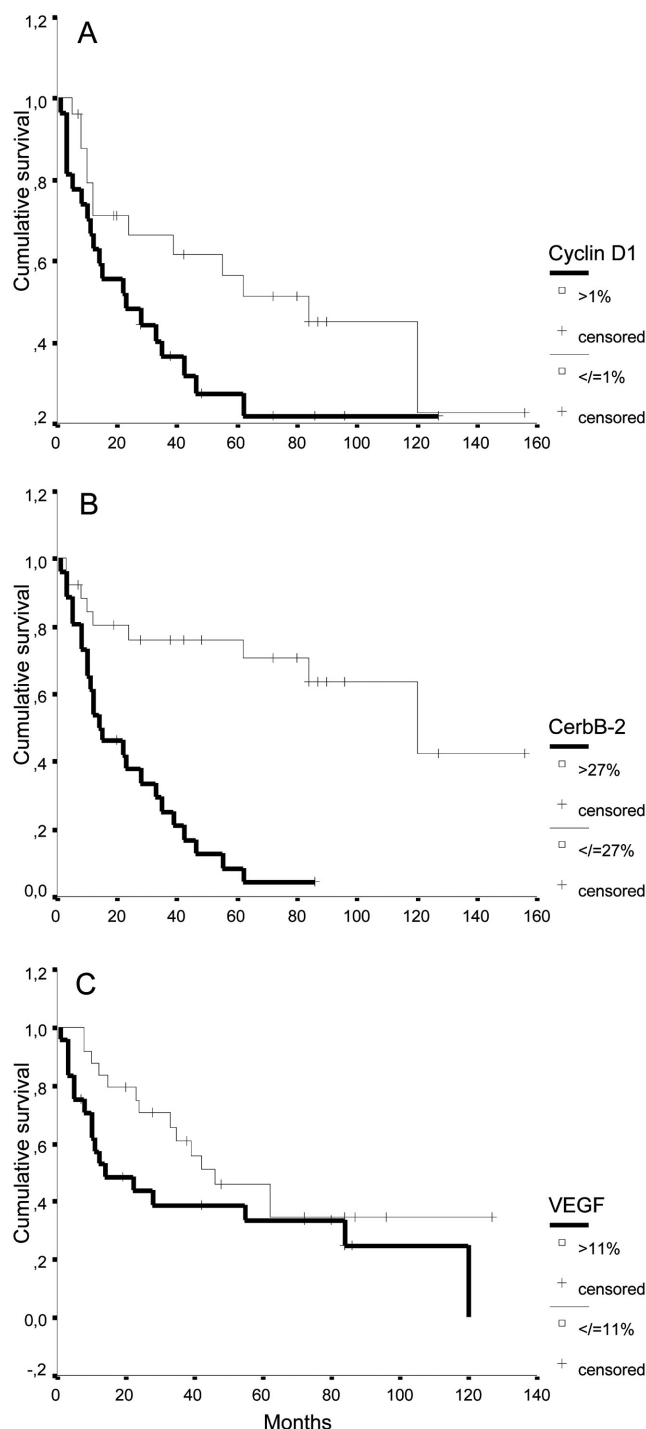
Fig. 2. Kaplan-Meier survival curve for patients with non-metastatic cancers and metastatic cancers grouped by **A**) Cyclin-D1 (p=0.04); **B**) cerbB-2 (p=0.04) and **C**) VEGF (p<0.01) grouped by low and high immuneexpression.

sites of distant metastatic disease include the brain, liver, adrenals, contralateral lung, bone and soft tissues. Metastases may present at the same time as the primary tumour or can occur much later, and they may be single or multiple, clinically silent or demand emergent diagnosis and treatment. About 60 percent of patients with lung adenocarcinoma have evidence of disseminated disease when they are first seen by a physician and unfortunately are rarely cured, despite the best efforts (Wingo et al., 1998).

The definition of the chemotherapy role in the treatment of this disease continues to progress and controversy exists concerning its role and benefit in advanced adenocarcinoma. Among the issues that have been raised with respect to the relative value of chemotherapy in patients with disseminated disease is the response rate of growth tumour, usually evaluated by serial radiological examination (Veale et al., 1993).

Recent interest has focused on the identification of different tumour cell features that may predict response to treatment, growth rate, metastases and death in patients with lung cancer. Tumour cell features may be classified by the mechanism of action that results in metastases, mainly growth rate and cell cycle regulation. Tumour and metastasis growth require growth-regulating factor, cell cycle-regulating and regulators of vasculogenesis proteins, such as c-erbB-2, Cyclin D1 and VEGF. In addition, the aggressiveness of a tumour depends on the features of neoplastic cells (proliferation and expression of oncogene). There is evidence that suggests that the interaction between Cell Cycle Regulator, Growth Factor Receptor and Vascular Endothelial Growth Factor influences the invasive and metastatic profile of the tumour (Gusterson et al., 1992; Keum et al., 1999; Shinkaruk et al., 2003; Shibuya, 2006). Differences in these aspects could, therefore, explain differences regarding the invasive and metastatic profiles among tumours of the same type and stage (Demarchi et al., 2000). Furthermore, lung adenocarcinoma displaying a wide spectrum of clinicopathological features with varying prognosis provide an interesting human tumour model to study the features of the disease through the interaction between Cell Cycle Regulator, Growth Factor Receptor and Vascular Endothelial Growth Factor.

In this study, the effects of the interaction between Cell Cycle Regulator (Cyclin D1), Growth Factor Receptor (c-erbB-2) and Vascular Endothelial Growth Factor (VEGF) in metastases vs corresponding primary cancers and metastatic vs non-metastatic cancers were presented. To our knowledge, the resulting effects of this interaction have not been previously documented. We found different tumour cell profiles in metastases, corresponding primary cancers, metastatic and non-metastatic cancers , thus suggesting that different cell clones control the invasive and non-invasive behaviour of the cancers. In fact, metastases and non-metastatic cancers expressed low c-erbB-2, Cyclin D1 and VEGF, while metastatic cancers expressed high c-erbB-2 with

an impact on survival of the patients.

Tumour growth and metastatic evolution depend on growth factor regulators (Blood and Zetter, 1990; Folkman, 1990). Data from our study demonstrated a direct interaction between the metastases and corresponding primary cancers. We found that Cyclin D1, cerbB-2 and VEGF were highly expressed in metastatic cancers, albeit low in non-metastatic cancers, indicating that Cyclin D1, cerbB-2 and VEGF are probably factors for growth, maintenance, progression and metastases of the tumours. These results suggest that actions of the Cyclin D1, c-erbB-2 and VEGF are similar in tumour progression and are supported by the pathway integration for metastatic progression, indicating that tumours with higher indexes of Cyclin D1, cerbB-2 and VEGF expression have the capacity to metastasize. The capacity of the neoplastic cells to metastasize in advanced tumours suggests a new tumour clone related to angiogenesis, and is supported by some studies related to c-erbB-2 and MMP9, a family of endopeptidase capable of degrading basement membranes and the extracellular matrix. MMP9 frequently are up-regulated in malignant disease, thus facilitating tumour growth, invasion and angiogenesis 30. Therefore, increased tumour expression of Cyclin D1 may be more a primary event related to cellular proliferation and differentiation, and c-erbB-2 and VEGF secondary events related to angiogenesis. Regardless of the mechanism, staining of the primary tumour for Cyclin D1, cerbB-2 and VEGF provides important prognostic information in adenocarcinomas of the lung and their hematogenic metastases.

Thus, for all these reasons, we should not be surprised to learn that Cyclin D1, cerbB-2 and VEGF provide important prognostic information about lung cancer, and our results now confirm the prognostic importance of Cyclin D1, cerbB-2 and VEGF in lung adenocarcinomas. Our results suggest that Cyclin D1, cerbB2 and VEGF used as categorical variables, provide more important prognostic information than does routine pathological staging. Thus, immunohistochemical staining for Cyclin D1, cerbB-2 and VEGF offers us the potential to guide the use of adjuvant chemotherapy in patients likely to fail treatment after surgical excision of cancers or their metastases.

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