

## Prognostic Value of *ERBB2* Amplification and Protein Expression in Small Cell Lung Cancer

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**OBJECTIVE:** Our objective was to evaluate *ERBB2* oncogene amplification using fluorescence in situ hybridization (FISH) and protein overexpression using immunohistochemical techniques, and to explore their possible prognostic value in a series of patients with small cell carcinoma.

**PATIENTS AND METHODS:** Included in the study were 99 patients with small cell tumors, classified in 2 broad groups: patients with limited or locally advanced disease and patients with disseminated disease. Material for study was obtained in 97% of the cases (96/99) by means of endoscopic biopsy and by tomography-guided needle biopsy in the remaining 3% (3/99). Survival was analyzed using the Kaplan-Meier method.

**RESULTS:** The 92 men (92.9%) and 7 women (7.1%) in the study had a mean (SD) age of 62.9 (10.4) years (range, 36-81 years); 39.4% (n=39) and 60.6% (n=60) of the subjects had limited and disseminated disease, respectively. *ERBB2* protein overexpression was observed in 26.3% of the patients (n=26), 15.4% (n=4) of whom had limited disease and 84.6% (n=22) of whom had disseminated disease ( $P=0.005$ ). Although mean survival was slightly longer for patients who were negative for *ERBB2* protein overexpression, the difference was not statistically significant. FISH identified gene amplification in 6.3% (1 in 16) of the studied cases (ratio, 2.3).

**CONCLUSIONS:** The protein product of the *ERBB2* oncogene is overexpressed in 33.3% of small cell lung carcinomas and is associated with the presence of disseminated disease. Further studies are necessary to evaluate the possible benefits of specific treatment.

Valor pronóstico de la expresión y amplificación genética de *c-erbB-2* en el carcinoma microcítico de pulmón

**OBJETIVO:** El propósito de nuestro estudio ha sido evaluar la sobreexpresión proteica de *c-erbB-2* mediante técnicas de inmunohistoquímica y la amplificación del oncogén mediante hibridación in situ fluorescente, en una serie de carcinomas microcíticos, correlacionándola con las posibles implicaciones pronósticas.

**PACIENTES Y MÉTODOS:** Se incluyó a 99 pacientes con tumores microcíticos clasificados en 2 grandes grupos: enfermedad limitada o localmente avanzada y enfermedad diseminada. El material para estudio se obtuvo mediante biopsia endoscópica en el 97% de los casos (96/99) o mediante punción guiada por tomografía computarizada en el 3% restante (3/99). La supervivencia se analizó con el método de Kaplan-Meier.

**RESULTADOS:** La media de edad  $\pm$  desviación estándar de los pacientes fue de 62,9  $\pm$  10,4 años (rango: 36-81). El 92,9% (n = 92) eran varones y el 7,1% mujeres (n = 7). Un 39,4% (n = 39) presentaba enfermedad limitada y el 60,6% (n = 60) enfermedad diseminada. La sobreexpresión proteica de *c-erbB-2* se observó en el 26,3% de los casos (n = 26), de los cuales un 15,4% (n = 4) presentaba enfermedad limitada y el 84,6% restante (n = 22) enfermedad diseminada ( $p = 0,005$ ). La media de supervivencia fue ligeramente mayor para los pacientes con *c-erbB-2* negativo que en aquéllos con *c-erbB-2* positivo, pero esta diferencia no fue estadísticamente significativa. La técnica de hibridación in situ fluorescente mostró amplificación génica en el 6,3% (1/16) de los casos estudiados, con un índice de 2,3.

**CONCLUSIONES:** El producto proteico del oncogén *c-erbB-2* se sobreexpresa en un 33,3% de los carcinomas microcíticos pulmonares y se asocia a la presencia de enfermedad diseminada. Son necesarios nuevos estudios para evaluar el posible beneficio del tratamiento específico.

**Key words:** Small cell carcinoma of the lung. *ERBB2* oncogene. Prognosis.

**Palabras clave:** Carcinoma microcítico de pulmón. *c-erbB-2*. Pronóstico.

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### Introduction

Small cell lung cancer represents approximately 15% to 25% of all malignant lung neoplasms.<sup>1</sup> The main features of this cancer are its neuroendocrine nature, aggressive biological behavior, and good response to chemotherapy.<sup>2</sup> Nonetheless, despite this responsiveness, fewer than 10%

of patients survive longer than 2 years after the commencement of treatment, and only 3% to 7% survive for 5 or more years.<sup>3</sup>

The *ERBB2* oncogene is the second member of the epidermal growth factor receptor family that codes for the p185 protein, which acts as a receptor tyrosine kinase that is associated with multiple signal transduction pathways.<sup>4</sup> Protein overexpression and amplification of the *ERBB2* gene have been encountered in various kinds of neoplasms, including lung tumors<sup>5</sup>; their expression is best established in breast cancer, however, where they have been observed in 15% to 40% of tumors<sup>6,7</sup> and where they are considered to indicate poor prognosis. In non-small cell lung tumors, particularly adenocarcinomas, the percentage of overexpression has been observed to vary between 20% and 30%<sup>8,9</sup>; the possible prognostic implications remain open to debate, however. A recent study of small cell lung cancer<sup>10</sup> used immunohistochemical detection to demonstrate *ERBB2* overexpression in 12 of 67 patients (17.9%) associated with a poor prognosis.

Use of the monoclonal antibody trastuzumab for the treatment of patients with metastatic breast cancer with *ERBB2* overexpression has raised hopes that it might be useful in treating other tumors that overexpress this protein.<sup>11</sup>

The aim of our study was to evaluate the prevalence of *ERBB2* oncogene amplification using fluorescent in situ hybridization (FISH), and protein overexpression using immunohistochemical techniques, and to explore their possible prognostic value in a series of patients with small cell carcinoma.

## Patients and Methods

### Patients

Included in the study were 99 patients with small cell lung cancer, diagnosed consecutively in the period 1997 to 2004. The data for these patients came from the records of the pathology department of Hospital Gregorio Marañón in Madrid, Spain. The tumors were classified according to the criteria of Stahel<sup>12</sup> into 2 broad groups: limited or locally advanced disease and disseminated disease. Included in the limited-disease group were tumors limited to 1 hemithorax, locally advanced disease included well-defined tumors with metastasis to contralateral hilar lymph nodes, and disseminated-disease included patients with distant metastasis.

Cancer stage was assessed by means of chest radiography; bronchoscopy with biopsy; chest, brain, and abdominal computed tomography; blood biochemistry tests, including differential cell counts; bone scintigraphy; and bone marrow biopsy. According to the TNM classification system, tumors up to stage IIIB represent limited disease, and those up to stage IV represent disseminated disease.

### Study Material

The study material was obtained by means of endoscopic biopsy during bronchoscopy in 97% (96/99) of patients and by means of computed tomography-guided needle biopsy in the remaining 3% (3/99). The material was fixed

in a 10% buffered formaldehyde solution, then embedded in paraffin and cut into 3- $\mu$ m sections. The samples were mounted on silane-treated microscope slides and incubated at 60°C overnight, after which they were deparaffinized with xylene, then rehydrated in successive baths of 100%, 96%, and 70% alcohol for 5 minutes each before being washed in distilled water for a further 5 minutes.

### Immunohistochemistry

*ERBB2* expression was determined using the HercepTest (Dako, Glostrup, Denmark), which uses a polyclonal antibody (AO485). The test was performed using material fixed in formaldehyde and embedded in paraffin. The test protocol unfolded in 2 stages. The first stage involved using a primary rabbit antibody to the human *ERBB2* protein, and the second stage involved the use of a secondary goat antirabbit antibody, with horseradish peroxidase as the reaction tracer enzyme. Diaminobenzidine was used as the chromogen and EnVision (Dako, Hamburg, Germany) as the visualization reagent. Staining was visualized and interpreted in accordance with the protocol described in the HercepTest instructions manual. The results were expressed as a value between 0 and 3+, with 0 and 1+ considered to be negative and 2+ and 3+ considered to be positive. Scoring was as follows: 0 indicated no staining; 1+ indicated faint and barely perceptible staining of at least 10% of tumor cells; 2+ indicated moderate and complete staining of the membrane in more than 10% of tumor cells; and 3+ referred to intense staining of the entire cellular membrane in more than 10% of tumor cells. The cases were independently assessed by 2 pathologists and interobserver agreement was evaluated using the  $\kappa$  statistic.

### Fluorescent in Situ Hybridization Technique

Fluorescent in situ hybridization (FISH) was used to detect *ERBB2* gene amplification in small cell carcinomas. PathVision (Abbott Molecular Inc, Wiesbaden, Germany) was used as being suitable for material fixed in formaldehyde and set in paraffin; moreover, it includes an orange-spectrum locus-specific identifier (LSI) DNA probe specific to the *ERBB2* gene (17q11.2-q12), and a green-spectrum centromere enumeration probe (CEP) 17, specific to the alpha satellite DNA sequence located at the centromere of chromosome 17 (17p11.1-q11.1). The preparation of material for FISH was identical to the procedure followed for the immunohistochemistry test, and the technique itself was applied in 3 stages, namely, denaturation of the DNA in the samples, hybridization, and post-hybridization washing. For interpretation purposes, results were recorded in a 2 $\times$ 2 table of the number of signals per nucleus for LSI *ERBB2* and CEP 17. Results for 60 tumor cell interphase nuclei were recorded, and the number of copies of *ERBB2* and CEP 17 were compared. Tumors with gene amplification had a signal ratio for *ERBB2* to CEP 17 of 2 or more, whereas normal tissue had a ratio of less than 2. Results close to the cutoff point (between 1.8 and 2.2) were observed and quantified using an alternative technique; if doubt persisted, the process was repeated.

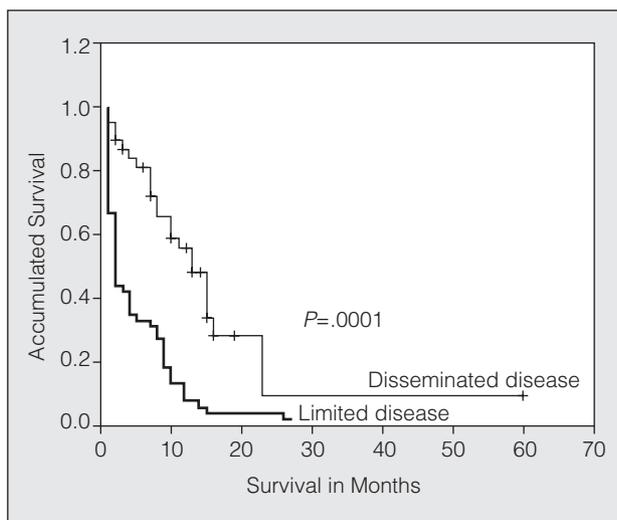


Figure 1. Survival curve according to cancer stage for patients with small cell lung cancer.

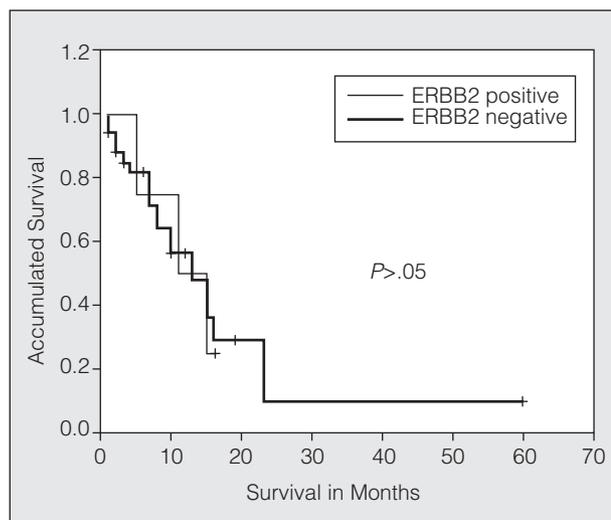


Figure 3. Survival curve for patients with the limited disease according to expression of ERBB2.

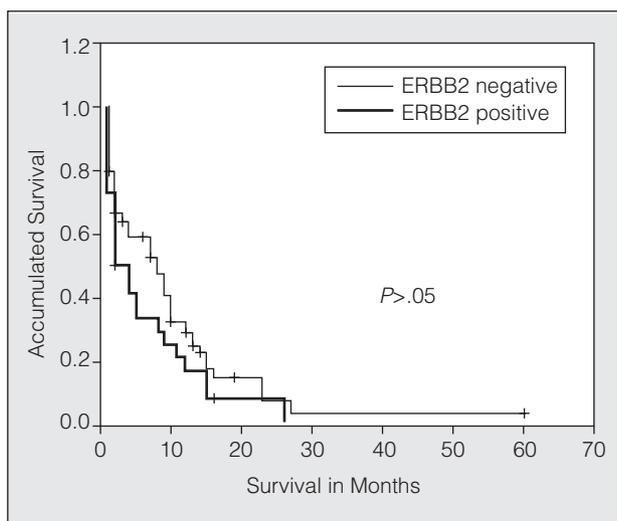


Figure 2. Survival curve according to immunohistochemical expression of ERBB2.

TABLE 1  
Patient Characteristics According to ERBB2 Expression

	ERBB2	
	Negative	Positive
No. of patients	73 (73.7%)	26 (26.3%)
Mean (SD) age, y	62.8 (9.9)	63.4 (11.9)
Sex		
Male	67 (91.8%)	25 (96.2%)
Female	6 (8.2%)	1 (3.8%)
Stage		
Limited disease	35 (47.9%)	4 (15.4%)
Disseminated disease	38 (52.1%)	22 (84.6%)
Survival		
1 y	28%	16%
2 y	7%	0%
Survival, mean (95% CI), mo	10 (7-14)	7 (4-10)

Abbreviation: CI, confidence interval.

### Statistical Analysis

Qualitative variables were expressed as absolute frequencies and percentages and compared using the  $\chi^2$  test or the Fisher exact test. Survival was calculated from the time of diagnosis to the date of death or the date of the last follow-up evaluation. The Kaplan-Meier method was used to analyze survival, with curves compared using with the log-rank test. Statistical significance was established at  $P < .05$ . Data was analyzed using the SPSS statistical package, version 11.5 (SPSS, Chicago, Illinois, USA) for Windows.

### Results

The study group was composed of 99 patients diagnosed with small cell lung cancer. A  $\kappa$  value of 0.99 indicated

agreement between 2 pathologists in regard to histopathologic evaluation.

The 2-year survival rate for patients with limited or locally advanced disease was 9% (mean 17 months; 95% confidence interval [CI], 9-14 months). The same rate for patients with disseminated disease was 0% (mean 5 months; 95% CI, 4-7 months) ( $P < .001$ , log-rank test) (Figure 1).

The clinical characteristics of the patients according to ERBB2 protein expression are shown in Table 1. ERBB2 expression (2+ and 3+) in small cell tumors was significantly associated with disseminated disease ( $P = .005$ ). On the other hand, no statistically significant differences ( $P > .05$ ) were found on comparing survival rates for the tumors that were positive and negative for ERBB2 (Figure 2).

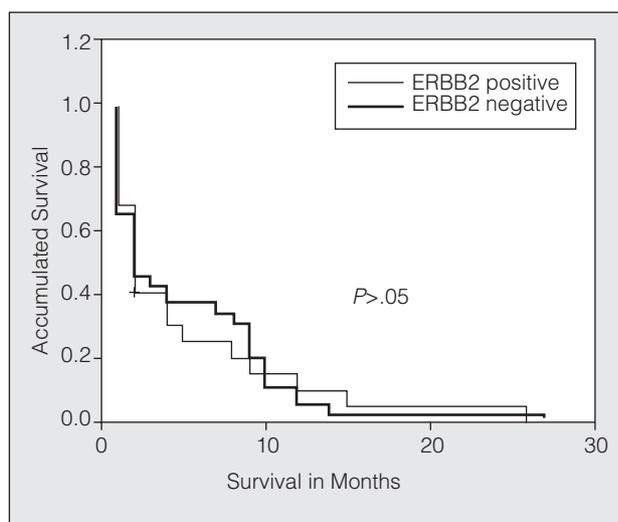


Figure 4. Survival curve for patients with the disseminated disease according to expression of ERBB2.

TABLE 2  
FISH Findings Distributed According to ERBB2 Protein Expression

ERBB2	
By Immunohistochemistry	FISH, n (%)
0	2 (6.1%)
+	17 (51.5%)
++	12 (36.4%)
+++	2 (6.1%) <sup>a</sup>

Abbreviation: FISH, fluorescent in situ hybridization.

<sup>a</sup>One case showed evidence of low-range amplification; total number of patients, 33.

Results for survival rates stratified by clinical stage and ERBB2 expression were as follows (all comparisons,  $P>.05$ ): for limited-disease patients with tumors positive for ERBB2, both the 1- and 2-year survival rates were 25% (mean survival, 12 months; 95% CI, 8-16 months); for limited-disease patients with tumors negative for ERBB2, the 1- and 2-year survival rates were 56% and 9%, respectively (mean survival, 17 months; 95% CI, 9-24 months); for disseminated-disease patients with tumors positive for ERBB2, the 1- and 2-year survival rates were 5% and 0%, respectively (mean survival, 5 months; 95% CI, 2-8 months); finally, for disseminated-disease patients negative for ERBB2, the 1- and 2-year survival rates were 5% and 0%, respectively (mean, 5 months; 95% CI, 3-7 months) (Figures 3 and 4).

Material suitable for FISH was obtained from 33 of the 99 patients (33.3%). The distribution of patients according to immunohistochemical expression of ERBB2 is shown in Table 2. A total of 16 of 33 patients (48.5%) were evaluable. Amplification was not evident in 15 patients (93.8%), and was demonstrated to be in the low range (an index of 2.3) in 1 patient (6.3%).

## Discussion

Overexpression of the protein of the *ERBB2* oncogene has been studied in numerous malignant tumors in different locations, but particularly in breast carcinomas,<sup>6,7,13</sup> colorectal cancer,<sup>14</sup> the female genital tract,<sup>15</sup> and, more recently, lung cancers.<sup>5,8,9,16</sup> A recent meta-analysis evaluating the prognostic value of ERBB2 expression for the survival of patients with lung cancer concluded that this might point to a poor prognosis for non-small cell lung cancer.<sup>17</sup>

In studies of small cell lung cancer cell-lines, neither messenger RNA nor the ERBB2 protein were expressed.<sup>18,19</sup> Sloman et al.<sup>20</sup> however, found overexpression in 19 of 28 patients (68%); for patients surviving less than 2 years overexpression was 93%. For 14 of 107 patients, Micke et al<sup>21</sup> reported ERBB2 expression as an independent predictor of shorter survival. Potti et al<sup>16</sup> observed ERBB2 expression in 29.5% of a series of 193 patients with disseminated disease; more recently, Canoz et al<sup>10</sup> reported expression of this oncoprotein in 12 of 67 patients (17.9%) with small cell cancer. In both these studies, overexpression of ERBB2 was demonstrated to predict a poor prognosis. Our results are consistent with those of both Sloman et al and Canoz et al, in that ERBB2 expression is significantly associated with the clinical stage of disseminated disease.

Protein overexpression in our series was observed in 26.3% patients, and, moreover, was significantly associated with advanced-stage tumors in patients with disseminated disease. These results should be interpreted with care, however, as most of our patients with an advanced-stage tumor presented with other clinical factors that would indicate poor prognosis.<sup>16</sup>

On analyzing patient survival according to ERBB2 expression and tumor stage, we observed that differences were not statistically significant for patients with the limited disease, even though mean survival was slightly greater in patients who were ERBB2 negative compared to patients who were positive. For patients with disseminated disease survival rates were similar, regardless of the expression of the ERBB2 oncoprotein. Consequently, we conclude that ERBB2 protein expression had no prognostic value for patient survival in our series.

Previous studies of ERBB2 expression in small cell lung cancers have been based on immunohistochemistry techniques, and we were therefore of the opinion that it was important to complement those studies by applying gene amplification using FISH techniques. Amplification of *ERBB2* was observed in 6.3% of the cases we studied. This low percentage may be attributable to the disease itself or to limitations of our study, such as that fact that all of the cases were diagnosed by means of endoscopic biopsy or needle biopsy guided by computed tomography. This obviously has a bearing on the suitability of tissue samples obtained for both immunohistochemistry and FISH. Trastuzumab, a monoclonal antibody that recognizes the ERBB2 receptor protein, is currently being investigated as a treatment for lung cancers with ERBB2 expression. The choice of detection method and the level of ERBB2 expression at which a potential therapeutic effect would be achieved have not yet been established for lung cancer.

In conclusion, the presence of a group of small cell tumors in which there is expression of the ERBB2 oncoprotein opens up the way to new therapeutic possibilities. We are also of the opinion that FISH is not the most appropriate technique for evaluating *ERBB2* gene amplification in samples obtained by means of small cell lung carcinoma bronchoscopy.

Prospective studies are needed to evaluate the prognostic value of ERBB2 expression in small cell lung cancer, with a view to defining high-risk groups that could merit receiving a more specific kind of treatment.

## REFERENCES

1. Elias AD. Small cell lung cancer: state-of-the-art therapy in 1996. *Chest*. 1997;112:251S-8S.
2. Ihde DC. Chemotherapy of lung cancer. *N Engl J Med*. 1992;327:1434-41.
3. Albain KS, Crowley JJ, Livingston RB. Long-term survival and toxicity in small cell lung cancer. Expanded Southwest Oncology Group experience. *Chest*. 1991;99:1425-32.
4. Hung McLau YK. Basic science of HER-2/neu: a review. *Semin Oncol*. 1999;26:51-9.
5. Pfeiffer P, Clausen PP, Andersen K, Rose C. Lack of prognostic significance of epidermal growth factor receptor and the oncoprotein p185HER-2 in patients with systemically untreated non-small-cell lung cancer: an immunohistochemical study on cryosections. *Br J Cancer*. 1996;74:86-91.
6. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. 1987;235:177-82.
7. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science*. 1989;244:707-12.
8. Tateishi M, Ishida T, Mitsudomi T, Kaneko S, Sugimachi K. Prognostic value of c-erbB-2 protein expression in human lung adenocarcinoma and squamous cell carcinoma. *Eur J Cancer*. 1991;27:1372-5.
9. Kern JA, Slebos RJ, Top B, Rodenhuis S, Lager D, Robinson RA, et al. C-erbB-2 expression and codon 12 K-ras mutations both predict shortened survival for patients with pulmonary adenocarcinomas. *J Clin Invest*. 1994;93:516-20.
10. Canoz O, Ozkan M, Arsav V, Er O, Coskun HS, Soyuer S, et al. The role of c-erbB-2 expression on the survival of patients with small-cell lung cancer. *Lung*. 2006;184:267-72.
11. Tapia C, Glatz K, Novotny H, Lugli A, Horcic M, Seemayer CA, et al. Close association between HER-2 amplification and overexpression in human tumors of non-breast origin. *Mod Pathol*. 2007; 20:192-8.
12. Stahel RA. Diagnosis, staging, and prognostic factors of small cell lung cancer. *Curr Opin Oncol*. 1991;3:306-11.
13. Jimenez RE, Wallis T, Tabasczka P, Visscher DW. Determination of Her-2/Neu status in breast carcinoma: comparative analysis of immunohistochemistry and fluorescent in situ hybridization. *Mod Pathol*. 2000;13:37-45.
14. Kapitanovic S, Radosevic S, Kapitanovic M, Andelinovic S, Ferencic Z, Tavassoli M, et al. The expression of p185(HER-2/neu) correlates with the stage of disease and survival in colorectal cancer. *Gastroenterology*. 1997;112:1103-13.
15. Zhang X, Silva E, Gershenson D, Hung MC. Amplification and rearrangement of c-erb B proto-oncogenes in cancer of human female genital tract. *Oncogene*. 1989;4:985-9.
16. Potti A, Willardson J, Forseen C, Kishor Ganti A, Koch M, Hebert B, et al. Predictive role of HER-2/neu overexpression and clinical features at initial presentation in patients with extensive stage small cell lung carcinoma. *Lung Cancer*. 2002;36:257-61.
17. Meert AP, Martin B, Paesmans M, Berghmans T, Mascaux C, Verdebout JM, et al. The role of HER-2/neu expression on the survival of patients with lung cancer: a systematic review of the literature. *Br J Cancer*. 2003;89:959-65.
18. Weiner DB, Nordberg J, Robinson R, Nowell PC, Gazdar A, Greene MI, et al. Expression of the neu gene-encoded protein (P185neu) in human non-small cell carcinomas of the lung. *Cancer Res*. 1990;50:421-5.
19. Schneider PM, Hung MC, Chiocca SM, Manning J, Zhao XY, Fang K, et al. Differential expression of the c-erbB-2 gene in human small cell and non-small cell lung cancer. *Cancer Res*. 1989; 49:4968-71.
20. Sloman A, D'Amico F, Yousem SA. Immunohistochemical markers of prolonged survival in small cell carcinoma of the lung. An immunohistochemical study. *Arch Pathol Lab Med*. 1996;120:465-72.
21. Micke P, Hengstler JG, Ros R, Bittinger F, Metz T, Gebhard S, et al. c-erbB-2 expression in small-cell lung cancer is associated with poor prognosis. *Int J Cancer*. 2001;92:474-9.