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THE PROGNOSTIC SIGNIFICANCE OF C-ERBB-2 ONCOPROTEIN IN LUNG CARCINOMAS

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ABSTRACT
We assessed the immunohistochemical expression of c-erbB-2 transmembrane receptor in 15 primary lung carcinomas using the polyclonal antibody anti-human c-erbB-2 oncoprotein. Tissue fragments routinely processed (formalin-fixed and paraffin-embedded) were then investigated for c-erbB-2 oncoprotein expression using the Avidin-Biotin Complex staining method. Tumor cells showed positive c-erbB-2 expression in 86.6% of non small cell lung carcinomas (NSCLC). Positive c-erbB-2 immunoreaction correlated with the advanced stage of the disease, with lymph node involvement and high metastasis rate. The results obtained suggest that tissue overexpression of c-erbB-2 protein in NSCLC characterizes a group of tumors with an aggressive clinical-biological behavior and poor prognosis; c-erbB-2 protein can be considered a useful tumor marker in the morphological diagnosis and monitoring the clinical behavior of these neoplasms.
Key words: lung carcinomas, immunohistochemistry, c-erbB-2 oncoprotein

INTRODUCTION

Tyrosin-kinase growth factor receptors are very much studied and they were classified in 9 different families according to the structure of coupling domains of the ligand – the extracellular domain and of the kinase – intracellular domain, as well as according to the nature of their activation ligands (Hirsch FR, 2002).

Type I of growth factor receptors includes the family of epidermal growth factor receptor (EGFR) that comprises some polypeptides involved in cancer development. The members of this family are: *EGFR* (also known as c-erbB-1), *HER2/neu* (also known as c-erbB-2), *HER-3* (c-erbB-3) and *HER-4* (or c-erbB-4) (Harris JR, 1996).

C-erbB-2 oncogene, named HER-2/neu, was identified for the first time in mice, as an oncogene activated by a point mutation, in a group of neuroblastomas clinically induced by ethyl-nitrosurea (McCann A, 1990; Kuniyuki O, 1994).

C-erbB-2 protooncogene, identified in human genome, is localized on chromosome 17q21 (McCann A, 1990; Toshihiro O, 1995) and it is transcribed in a RNA of 4.5 kilobases (Kb) (Harris JR, 1996; McCann A, 1990); the protein coded by this gene is a transmembrane glycoprotein (Kuniyuki O, 1994; Toshimasa K, 1994) with tyrosin-kinase activity and molecular weight of 185 kDa (McCann A, 1990), known as p185.

Comparison between human c-erbB-2 gene product and EGFR shows a homology of approximately 50% in aminoacid sequence. According to the aminoacid sequence inferred for c-erbB-2 protein, it was postulated that c-erbB-2 encodes a transmembrane protein similar to EGFR.

Although many protooncogenes are amplified in various types of neoplasias, their role in the development of specific human malignant tumors is not clear. Increasing evidence show that amplification and hyperexpression of a protooncogene can produce in vitro cell transformation and they can participate to the neoplastic process (McCann A, 1990).

The mechanism that leads to overexpression of c-erbB-2 in lung cancer is not known. Gene amplification described in mammary cancer is not frequent in lung cancer, suggesting the involvement of other transcriptional and post-transcriptional regulating mechanisms. Although there are many studies that describe the presence of factors stimulating c-erbB-2 protein activity, the nature of its good functioning is not well understood (Toshihiro O, 1995).

The function of c-erbB-2 protein in normal growth and tumor cell differentiation is still unclear; this is also true for its role in tumor development. It seems possible for a gene that encodes a growth factor receptor – when it is overexpressed, to offer a growth advantage to cells that express it. Alternately, alteration even of gene product can lead to a critical modification of the receptor protein. A single point mutation in the transmembrane domain of the protein encoded by the oncogene seems to be enough for the gene to acquire transformation capacity.

EGFR homology observed in the aminoacid structure of c-erbB-2 oncoprotein suggests that c-erbB-2 receptor could behave similar to EGFR; so, EGFR can also be considered a potential growth stimulator (McCann A, 1990).

C-erbB-2 oncogene amplification, which encodes a receptor for the growth factor related to EGFR, was identified for the first time by King and colab. (1985) in mammary cancer (Meden H, 1994) and proved to be a significant prognostic parameter for survival and relapse rates.

Yokoto J. (1986) and Tal M. (1988) reported specific amplification of c-erbB-2 or neu gene in carcinoma with

glandular epithelial origin (adenocarcinomas). Furthermore, the authors sustain that gene magnification is very tightly related to the advanced stage of the disease, showing even that there are clues for the cases in which amplification is bigger than five times, and that it can be correlated with relapses and reduced survival (Meert AP, 2003).

The aim of this study is to determine the frequency of c-erbB-2 oncogene overexpression in lung carcinomas, to evaluate its correlation with histological subtype and clinical parameters, as well as to assess the prognostic significance of p185 protein, encoded c-erbB-2.

MATERIAL AND METHOD

We assessed the expression of c-erbB-2 oncoprotein on tissue sections from 15 patients (13 men and 2 women) with lung cancer, using the immunohistochemical method of staining. The age of patients varied between 40 and 71 years old, with a mean age of 57 years; all patients were diagnosed and treated in the Thoracic Surgery Clinic of the Town Hospital and "Victor Babeş" Hospital from Timişoara.

The histological material – obtained through optic fiber bronchoscopy (5 cases), at necropsy (1 case) or from tissues surgically resected before radio- or chemotherapy (9 cases) – was fixed according to routine procedure (in 10% formalin for 24 hours) and embedded in paraffin, then stained with hematoxylin-eosin and immunohistochemically for c-erbB-2 oncoprotein.

Tumor stages were established according to the International Staging System for lung cancer: 3 patients had stage II of the disease, 8 patients stage III and 4 patients stage IV; 6 of the 15 patients showed distant metastasis discovered during the surgery or postoperatively at the histopathological exam; lymph nodes were involved in 7 cases and tumor relapses were found in 4 cases. Sections stained with hematoxylin-eosin were examined and histologically classified according to WHO classification of lung tumors, and the malignity grade was determined after Broders.

Immunohistochemical staining was made on sections 4-5μm thick, using the Avidin Biotin Complex technique (ABC). Overexpression of c-erbB-2 oncoprotein was detected using a polyclonal antibody that reacts with the intracellular domain of the protein.

The antibody anti-human c-erbB-2 protein (Dako) used for immunostaining is a rabbit polyclonal antibody, oriented against a synthetic peptide from the intracytoplasmic domain of c-erbB-2 oncoprotein. This antibody recognizes effectively c-erbB-2 protein on formalin-fixed and paraffin-included sections. After pre-treatment (boiling for 30 minutes at 80°C in a microwave oven) and blocking of endogenous peroxidase, sections were incubated over night at 4°C with the primary antibody; a dilution of 1:200 was applied, this concentration of the antibody proving to be optimal after staining assays with serial dilutions of the antibody. The next day, sections were incubated for 30 minutes with the second antibody (rabbit biotinated anti-IgG) and for another 30 minutes with Avidin-Biotin Complex reagent. For visualization we used 3,3' – diaminobenzidine tetrahydrochloride (DAB). For the positive and negative control we included the following sections: for the negative control, the buffer replaced the primary antibody on tumor sections and tissue fragments from 2 patients with benign lung diseases; as positive control sample we included a mammary gland carcinoma, intensely positive for c-erbB-2 oncoprotein.

Quantification of c-erbB-2 immunoreaction

P185 overexpression appears as a brown staining of the cell membrane. The staining reaction was considered positive when in cytoplasmic membrane, in cytoplasm or in both, dark brown deposits were visible.

We appreciated positivity as the level of c-erbB-2 protein expression, based on staining intensity, as follows:

- (negative): no cancer cell stained;

+/- : weak or ambiguous staining in less than 5% of neoplastic cells;

+ (positive): less than 50% of tumor cells stained;

++ (intensely positive): over 50% of neoplastic cells proved positive or intensely positive immunostaining.

In the absence of a standardized method of interpretation for c-erbB-2 immunostining, the studied cases were noted on the basis of their staining intensity. Thus, we considered as tumors with c-erbB-2 protein overexpression only those cases that presented well defined membrane positivity.

RESULTS

Using the Dako polyclonal anti-human c-erbB-2 oncoprotein antibody, we examined immunohistochemically 15 primary lung tumors in order to identify those neoplasias with overexpression of HER-2/neu oncoprotein. Positivity for this presumed transmembrane protein appeared as a granular brown staining localized predominantly at the level of the cell membrane. We identified c-erbB-2 positive immunoreaction in 13 of the 15 cases analyzed (86.6%) (Table 1), the rest of the cases (13.4%) being negative for this protein. 3 tumors stained weakly positive or ambiguous (+/-); in 4 cases, the immunoreaction was present in less than 50% of tumor cells (+), and other 6 cases proved to be intensely positive (++), staining being present in more than 50% of neoplastic cells. In the majority of tumors with positive immunoreactions, the cell membrane presented staining with heterogeneous intensity, some areas being more intensely colored than others. Furthermore, we remarked an evident heterogeneity of intratumor staining – tumor areas with intensely positive membranes included in widely negative tumor areas. In 4 cases we observed a conspicuous cytoplasmic staining, its significance being differently interpreted.

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Variable	Total no. of cases	c-erbB-2 immunostaining				No. positive cases (%)
		-	+/-	+	++	
Control tissues	2	2	0	0	0	0
NSCLC	15	2	3	4	6	**13 (86.6%)**
ADK	8	1	2	0	5	**7 (87.5%)**
SCC	5	1	1	2	1	**4 (80%)**
LCC	2	0	0	2	0	**2 (100%)**
Sex: men	13	2	2	3	6	**11 (84.6%)**
women	2	0	1	1	0	**2 (100%)**
Age: ≤60	7	0	3	2	2	**7 (100%)**
>60	8	2	0	2	4	**6 (75%)**
Smoking	11	2	0	2	7	**9 (81.8%)**
Non-smoking	4	0	1	1	2	**4 (100%)**
Stage: II	3	1	2	0	0	**2 (66.6%)**
III + IV	12	1	1	4	6	**11 (91.6%)**
Grade: G2	7	1	1	2	3	**6 (85.7%)**
G3	8	1	2	2	3	**7 (87.5%)**
Lymph nodes: N0	8	2	2	2	2	**6 (75%)**
N1+N2	7	0	1	2	4	**7 (100%)**
Distant metastases: M0	9	2	3	2	2	**7 (77.7%)**
M1	6	0	0	2	4	**6 (100%)**

NSCLC: non small call lung carcinoma; ADK: adenocarcinoma; SCC: small cell carcinoma; LCC: large cell carcinoma

Comparing the clinical-morphological characteristics of patients after dividing them in 4 groups (according to c-erbB-2 immunostaining), we did not establish a significant correlation neither between c-erbB-2 immunoreactivity and sex, age or smoking, nor between p185 positive immunoreactions and the degree of tumor malignity (Table 1). Table 1 shows the relationship between c-erbB-2 protein positivity rate and clinical-pathological features of patients.

But, we noted a relationship between c-erbB-2 positivity and clinical stage: 2 of the 3 tumors found in stage II stained weakly or ambiguous, while 11 of the 12 NSCLC found in stages III and IV showed positive p185 immunoreaction. The percent of p185 positive cases varied based on the biological type of the tumor. C-erbB-2 protein overexpression was found immunohistochemically in 13 of the 15 NSCLC (86.6%) examined, high levels of c-erbB-2 being expressed especially by adenocarcinomas (Fig. 1a-c). As compared to adenocarcinomas, the other histological types – epidermoid carcinomas (Fig. 2a, b) and undifferentiated large cell carcinomas (Fig. 3) presented staining in less than 50% of tumor cells, with a much weaker staining intensity. We did not include in our study small cell lung carcinomas because after staining assays, none of the evaluated cases proved immunoreactive; yet, we observed (on pieces obtained by lung biopsy) a staining of squamous metaplastic lung epithelium, overlying the tumor.

In what concerns lymph nodes involvement, most of NSCLC patients with ganglion metastasis belonged to the groups with positive and intensely positive c-erbB-2 immunoreactions; distant metastasis and tumor relapses were also observed between the NSCLC cases with high levels of c-erbB-2 expressions.

We evaluated the prognostic significance of c-erbB-2 expression by comparing survival of patients after dividing them into 2 groups:

1. The group of patients with negative of weakly positive (– or +/-) c-erbB-2 staining, in which under 5% of tumor cells stained weakly or ambiguous, and
2. The group of patients with positive or intensely positive (+/++) c-erbB-2 expression, immunostaining being present in over 5% of neoplastic cells.

After a mean follow-up of 39 months (between 38 and 40 months), we observed that 2 patients from the group with negative or weakly positive c-erbB-2 staining had a lifetime duration significantly longer than the patients from the other group, where mean survival (estimated at 38 months) was not attained at the moment of the last control. The group of patients with positive or

intensely positive c-erbB-2 tumors had a mean lifetime of 9 months (between 2 and 21 months).

Discussions and conclusions

Non-small cell lung cancers (NSCLC) are associated with high mortality world-wide. C-erbB-2 (HER-2) is a transmembrane receptor with tyrosine-kinase activity made up of an intracellular, a transmembrane and an extracellular domain (Cheng CM, 2005).

C-erbB-2 protein was detected in patients with various forms of carcinoma, its usefulness as a potential tumor marker being studied (Toshihiro O, 1995). Using the c-erbB-2 anti-serum (Dako) we identified lung tumors with overexpression of this transmembrane receptor, also evaluating the utility of oncoprotein tissue expression as tumor marker in such neoplasias.

In our study, c-erbB-2 protein overexpression was demonstrated by means of immunohistochemistry in 13 (86.6%) of the 15 lung carcinomas examined. The immunohistochemical reactivity pattern highlighted the localization of c-erbB-2 protein in the cell membrane, but also in the cytoplasm of cells under the form of dark brown deposits. C-erbB-2 oncoprotein positivity, easily identifiable as a brown granular color, localized predominantly in cell membranes, sustains the idea that c-erbB-2 protein is a transmembrane protein (Cornianu M, 1997); previous studies indicated that this membrane staining is correlated with c-erbB-2 gene amplification. The observation that in most c-erbB-2 positive tumors, cell membranes presented a staining of heterogeneous intensity, with more intense areas dispersed in widely negative areas is of particular significance. The heterogeneous distribution of staining, also remarked in other studies, suggests that immunostaining can identify small groups of tumor cells with a high level of c-erbB-2 protein expression, cell populations that at a DNA analysis could go undetected (McCann A, 1990).

It is considered that cytoplasm positivity – observed by us in 4 cases (++), represents a crossed reaction with mitochondrial protein with different molecular weight. Brumm C et al. (1990) affirm that cytoplasm product does not represent a form of c-erbB-2 protein (Coombs LM, 1993), while De Potter CR et al. (1989) reported a c-erbB-2 immunoreactive protein of 155 kDa in the cytoplasm of malignant normal cells. The recent observations of Brumm C et al. (1990) sustain that loss of cell differentiation is associated with high c-erbB-2 cytoplasm immunoreactivity (Coombs LM, 1993).

Our results disclose a different expression of c-erbB-2 protein in histological subtypes of NSCLC, adenocarcinomas expressing the highest levels of the protein. This observation is well correlated with the studies of Tal M (1988) and Yokoto J (1986) who reported specific amplification of c-erbB-2 gene, as well as overexpression of c-erbB-2 protein in squamous cell lung carcinomas (Toshihiro O, 1995); in our study we observed a positive c-erbB-2 immunoreaction in 4 cases, but with a much weaker staining intensity than in adenocarcinomas.

For assessing prognostic significance of c-erbB-2 immunostaining in correlation with other indicators of poor prognosis, we considered clinical stage, histological grade of differentiation, lymph node involvement and metastasis potential. The high percentage of p185 positive cases in tumors stages II and IV suggests a possible association of c-erbB-2 overexpression with the advanced stage of the disease. Most authors found a c-erbB-2 positive imuunoreaction more frequently in the cases with high grade of malignity, an observation that was not confirmed in our cases. C-erbB-2 gene amplification, as well as the elevated level of protein expression, was associated with the low degree of differentiation, with the presence of ganglion metastases and short survival in a lot of human carcinomas (Toshihiro O, 1995). In our study, elevated tissue levels of c-erbB-2 were expressed especially by tumors with lymph node involvement and with a high potential of relapse and metastasis.

Unlike the big number of studies on mammary cancer, literature data about lung cancer are scarce, a limited number of observations about c-erbB-2 oncoprotein expression or/and oncogene amplification being available. In the paper of Toshihiro Osaki (1995), c-erbB-2 oncoprotein expression was observed in 38 of lung adenocarcinomas and correlated with a severe prognosis; many studies that used multivariate analysis affirm that c-erbB-2 expression is one of the independent factors of poor prognosis in patients with lung adenocarcinomas. In other studies on pulmonary adenocarcinomas (Kern JA, 1990; 1994), patients with c-erbB-2 positive tumors had a poorer prognosis than those with c-erbB-2 negative tumors (Toshihiro O, 1995).

On the material that we examined, it seems that the cases with c-erbB-2 negative or weakly positive expression (- or +/-) have a better prognosis than the cases that express elevated c-erbB-2 levels. Because of the limited group of patients studied and the short periods of follow-up, we are not able to further comment aspects about prognosis and survival rate. However, our results suggest that c-erbB-2 protein overexpression determined immunohistochemically in lung carcinomas other than with small cells ("non small cells carcinomas"), characterizes a group of tumors with aggressive clinical-biological behavior and poor prognosis; c-erbB-2 protein can be considered a useful tumor marker in the morphological diagnosis and in monitoring the clinical behavior of these neoplasms.

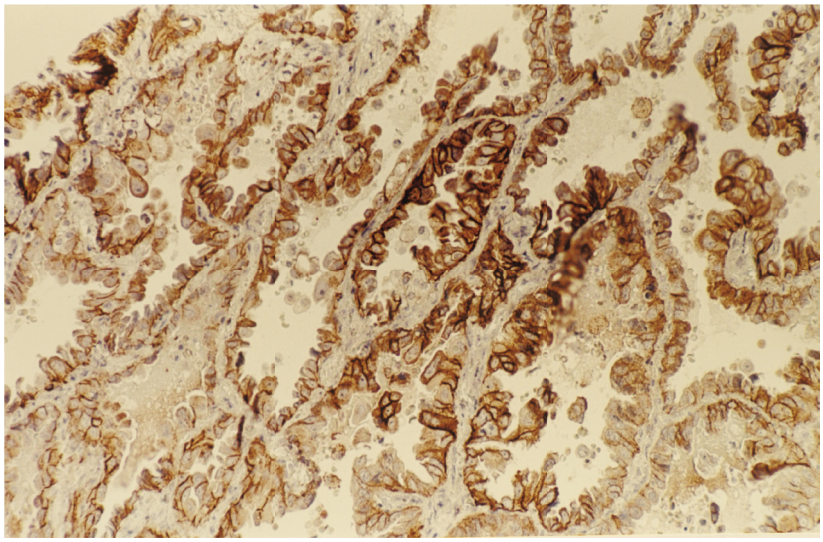

Fig. 1a. Lung adenocarcinoma with c-erbB-2 intensely positive expression (++) with membranar staining pattern. ABC x 200

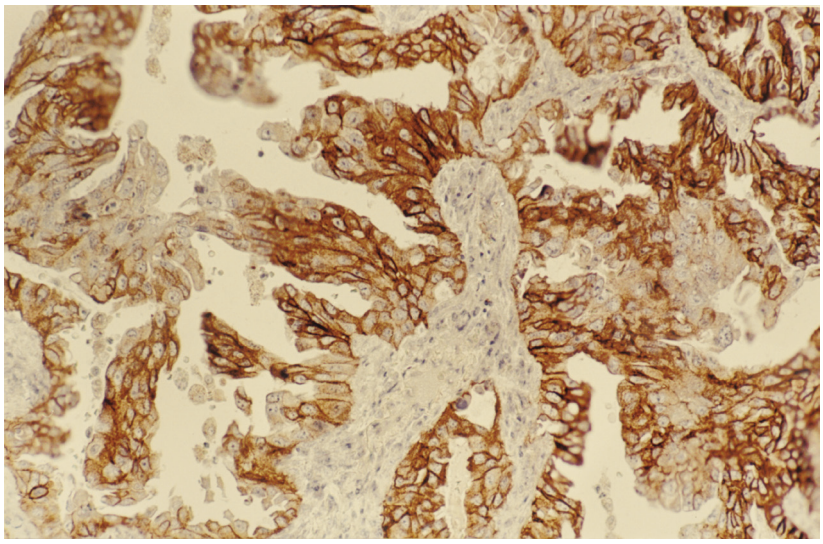

Fig. 1b. Lung adenocarcinoma with intense c-erbB-2 expression. ABC x 200

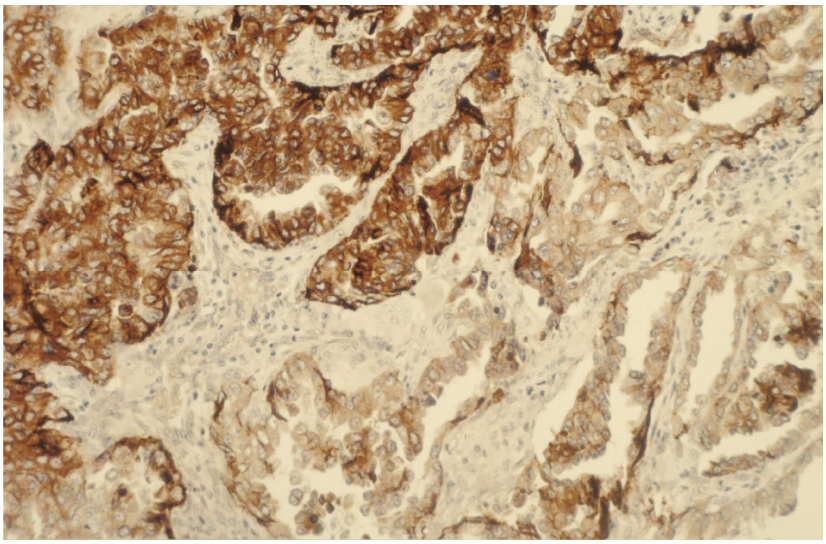

Fig. 1c. Lung adenocarcinoma with intense c-erbB-e expression in poorly differentiated areas. ABC x 200

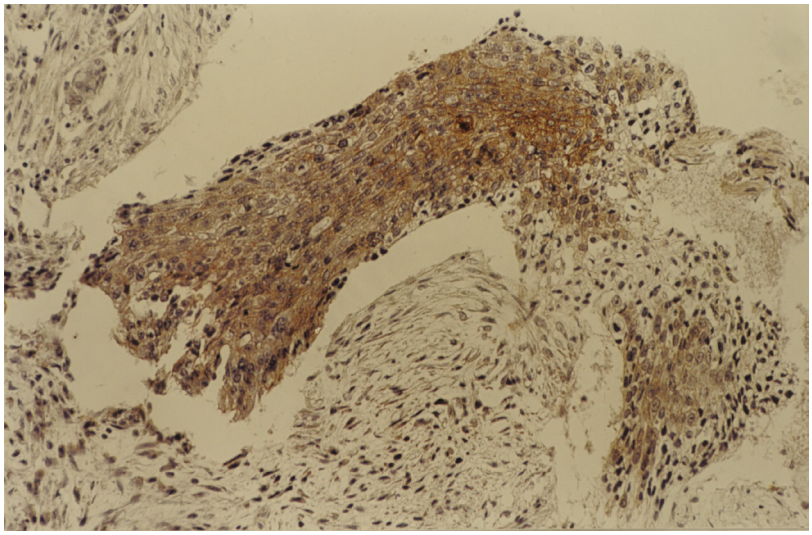

Fig. 2a. Epidermoid non-keratinized carcinoma. Positive c-erbB-2 immunoreaction of intermediate intensity (+). ABC x 200

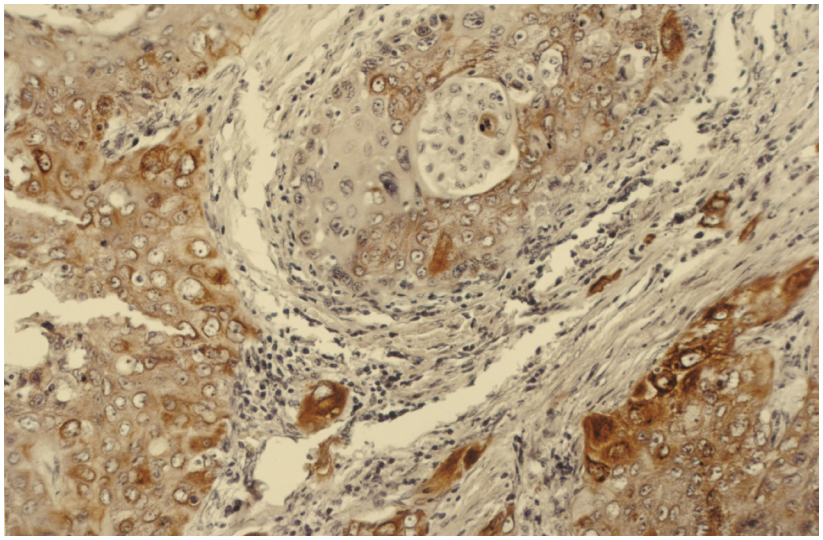

Fig. 2b. Epidermoid keratinized carcinoma with keratosic pearls; c-erbB-2 heterogeneous expression. ABC x 200

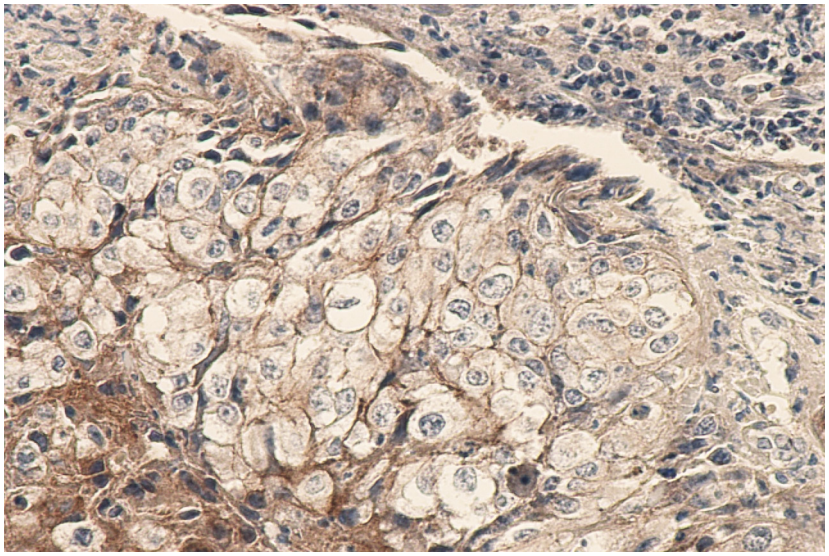

Fig. 3. Undifferentiated lung carcinoma with large cells. C-erbB-2 heterogeneous immunoreaction with membranar staining patter. ABC x 200

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