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Ploidy, expression of *erb*B1, *erb*B2, P53 and amplification of *erb*B1, *erb*B2 and *erb*B3 in non-small cell lung cancer

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Ploidy, expression of erb*B1,* erb*B2, P53 and amplification of* erb*B1,* erb*B2 and* erb*B3 in non-small cell lung cancer. N. Reinmuth, B. Brandt, W-P. Kunze, K. Junker, M. Thomas, R. Achatzy, H.H. Scheld, M. Semik. ©ERS Journals Ltd 2000.*

ABSTRACT: The aim of this study was to assess the prognostic value of deoxyribonucleic acid analysis, expression of *erb*B1, *erb*B2 and P53, and amplification levels of *erb*B1, *erb*B2 and *erb*B3 in non-small cell lung cancer (NSCLC).

Consecutive patients with NSCLC who underwent treatment with curative intention (118) were included. In 108 cases, the cell cycle was analysed using flow cytometry and double-staining with propidium iodide and anticytokeratin. In another 108 cases, expression of *erb*B1, *erb*B2 and P53 was assessed immunhistochemically. Amplification of the *erb*B family was determined in the tumours of 53 patients using double-differential polymerase chain reaction.

Of the tumours, 81% were aneuploid and 14% showed positive staining for *erb*B1, 18% for *erb*B2 and 41% for P53. There were normal mean gene copy numbers in 86% for *erb*B1, 94% for *erb*B2 and in 96% for *erb*B3. No significant correlations were noted between *erb*B1, *erb*B2 and P53 expression, ploidy status and tumour stage. In a Cox regression model, only tumour stage was shown to be prognostically significant.

It seems that ploidy and expression status of *erb*B1, *erb*B2 and P53 are not prognostic parameters in non-small cell lung cancer. Amplification of the *erb*B family does not seem to be a frequent event in non-small cell lung cancer.
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Lung cancer is the leading cause of cancer death in the USA and Europe [1, 2]. Non-small cell lung cancers (NSCLCs) are a biologically heterogeneous group of cancers with varying clinical presentations. Therefore, groups of patients with different prognosis need to be identified for treatment planning and outcome. The most accurate prognostic factor for patients with NSCLC is the tumour, node, metastasis (TNM) staging system, which is based on the anatomical extent of the tumour disease [1, 2]. However, even within the same TNM subset, there are obvious disparities in outcome. In patients with operable primary tumours, distant failure is a frequent event. Of patients thought to have stage I or II disease, disease will recur within 3 yrs after a curative operation in ~40–50%, which indicates that these patients cannot be cured by surgery alone [1]. Therefore, it appears that better techniques are needed for more accurate staging and identification of those patients at risk of disease relapse [3].

In recent years, several molecular markers have been detected in NSCLC [2, 4]. Malignancy results from deoxyribonucleic acid (DNA) damage, which modifies the normal checks and balances that regulate cellular proliferation and differentiation. Genetic alterations include the change of proto-oncogenes into oncogenes and the loss of tumour suppressor genes [5]. Conflicting information has been given regarding the relationship between overexpression of *erb*B1, *erb*B2, P53, ploidy, clinicopathological parameters and outcome [2, 6]. Most published series have been retrospective studies that lacked accurate methods, analysed only one molecular marker and performed inappropriate analysis of laboratory results [6, 7]. Furthermore, the molecular changes that lead to overexpression of the *erb*B family in NSCLC are not satisfactorily known. Therefore, a prospective study was conducted to investigate possible relations and the prognostic value of ploidy, expression of *erb*B1, *erb*B2 and P53, and amplification of *erb*B1, *erb*B2 and *erb*B3.

Patients and methods

Patient selection

Patients with primary NSCLC admitted between February 1993 and December 1994, who underwent surgical staging and R0 resection, were included in the study. An operation was considered to be R0 resection if the entire tumour was removed with microscopic negative surgical margins, an R1 resection if microscopic surgical margins were positive, and a R2 resection if gross tumour was left behind. Histological types of tumour were classified according to World Health Organization criteria [8]. The postsurgical stage of each tumour was determined according to the International Union Against Cancer [1]. Follow-up data were obtained at 1 and 2 yrs after

treatment by the primary care physician. Patients who died from any cause, excluding lung cancer, were censored.

Deoxyribonucleic analysis

For each patient, a sample of the tumour was submitted for DNA quantification at the time of operation. The sample was either directly analysed or frozen at -70°C for later analysis. Tumour cells were harvested using a scalpel, scraping along the fresh cut surface. The cells were suspended and fixed by the slow and dropwise addition of cold (-20°C) 70% ethanol while agitating on a vortex mixer to give a final ethanol concentration of 50%. After fixation and filtration through a nylon sieve (40 mm mesh width), the filtrate was washed in phosphate-buffered saline containing 5% bovine serum/albumin (Behring, Marburg, Germany). The suspension was then diluted to 1×10^6 cells·mL^{-1} and 20 µL of this suspension was mixed with 20 µg fluorescein isothiocyanate labelled anticytokeratin (Dako MNF 116; Dako Hamburg, Germany). For DNA staining, the normal procedure of ribonuclease treatment (R4875; Sigma, Deisenhofen, Germany) and propidium iodide (Sigma; 50 µg·mL^{-1}) staining was performed. The intact anticytokeratin and propidium iodide dual-stained cell suspensions were analysed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA) running Cell-fit software. The tumour samples were analysed at a low flow rate, with a mean measurement of 50,000 events per sample.

Histogram analysis

Keratin-positive cells were compared to keratin-negative cells as a diploid control. Every G0/G1 peak consisting of ≥15% of all cells measured showing at least a discrete G2/M peak was seen to represent a stem line of the tumour. The variation coefficient (CV) was assessed at every analysis. At the first ten flow cytometric analyses, the diploid control group was assumed to be represented by the first peak in the histogram without anticytokeratin staining because of an older software version.

Tumours were classified as aneuploid only if the tumour had more than one stem line or the G0/G1 modal of the tumour was more than three-fold greater than the CV of the G0/G1 modal of the control. The ploidy index was defined as the ratio of the carcinogenic G0/G1 modal to the G0/G1 modal of the control multiplied by two. In cases involving several stem lines, all calculations referred to the stem line containing the greatest number of tumour cells.

Gene amplification analysis

The gene dosages of *erb*B1, *erb*B2 and *erb*B3 were examined in frozen tumour tissue from 53 patients using double-differential polymerase chain reaction (PCR) (ddPCR) with leukocytes as negative control and superoxide dismutase 2 as the reference gene as described by BRANDT *et al.* [9]. The tumour samples were to be transported to the laboratory using isolated boxes transported containing dry ice and then stored again at -70°C until analysis. The DNA was extracted using a nucleic acid extraction kit (Isoquick; Progen, Queensland, Australia). The concentration and purity of the DNA was calculated by absorbance of ultraviolet light measurements. After adjustment for absorbance at 320 nm, the ratio of the absorbance at 260 to 280 nm was used to determine DNA concentration.

Protein expression analysis

Immunohistochemical analysis was performed on representative paraffin blocks from each resected tumour. The staining procedure was as follows: 1) paraffin microtome sectioning (3–4 µm) and slide labelling; 2) deparaffinization with xylol and ethanol; 3) antigen retrieval by heating four times in citrate buffer (pH 6.0; 0.1 M) in a microwave for 5 min (full power; 1000 watts microwave) for P53 investigation or with heated target unmasking fluid (TUF) buffer (95°C) (Dianova, Hamburg, Germany) for *erb*B1 and *erb*B2 assessment; 4) incubation with the (rabbit) polyclonal primary antibody (P53: CM-1 (Medac, Hamburg, Germany), *erb*B1: Ab-4 (Oncogene Science, Mineola, NY, USA), and *erb*B2: A0485 (Dako, Glostrup, Denmark)); 5) incubation with mouse antirabbit antibody and rabbit antimouse antibody; 6) development with alkaline phosphatase antialkaline phosphatase complex (Dako) and neu-fuchsin staining complex (Schmidt, Köngen, Germany); and 7) counterstaining with haematoxylin.

Slide evaluation

As negative control, the primary antibody was omitted. Sections from tumours with known *erb*B1, *erb*B2 or P53 overexpression served as positive control. *Erb*B1 and *erb*B2 slides were classified as positive if clear cytoplasmatic membrane staining of ≥5% of the malignant cells was observed. Clear nuclear staining of at ≥5% of the malignant cells was indicative of P53 accumulation. Minimal sporadic staining of an occasional isolated tumour cell and diffuse cytoplasmatic staining were interpreted as nonspecific.

Statistics

All patients and specimens received different codes which were revealed only during statistical analysis. The log rank test was used to compare the different subgroups with respect to cancer-specific survival. The Kaplan-Meier method was used to estimate the probability of survival as a function of time. The Cox proportional hazards modelling technique was used to identify which independent factor had a significant influence on overall survival.

Results

In 118 cases, NSCLC was histologically confirmed and the tumour completely resected with sufficient tumour-free periphery of the resection margin (table 1). Flow cytometric analysis was performed on 108 tumours. Eighty-seven (81%) tumours were aneuploid and 21 (19%) diploid. The mean CV of the G0/G1 peak of the diploid reference population was 6.48% (range 2.36–14.1%). Fifty-nine (55%) tumours had only one stem line, 48

Table 1. – Clinical parameters of the 118 patients included in the study

Male/female	108 (92)/10 (8)
Age distribution yrs	
<40	1 (1)
40–49	7 (6)
50–59	38 (32)
60–69	59 (50)
>69	13 (11)
Mean age yrs	62
Histology	
Squamous cell cancer	48 (41)
Adenocarcinoma	31 (26)
Undifferentiated large cell carcinoma	39 (33)
Tumour stage	
I	76 (64)
II	9 (8)
IIIA	26 (22)
IIIB	5 (4)
IV with ipsilateral lung metastases	2 (2)
Surgical treatment	
Lobectomy	74 (63)
Bilobectomy	7 (6)
Pneumectomy	37 (31)
Histological grade of differentiation	
1	6 (5)
2	35 (30)
3	74 (63)
4	1 (1)
Unknown	2 (2)

Data are presented as n (%).

(44%) had two and one (1%) had three. Most tumours showed a DNA index of the first DNA peak of 1.8–2.2 (43 cases), illustrated in figure 1. The distribution of stage, histology, tumour differentiation grade and age was not significantly different between the aneuploid and diploid groups.

In 108 tumours, expression of *erb*B1, *erb*B2 and P53 was assessed immunohistochemically. In one case, expression of P53 could not be determined because of insufficient quantity of tumour. No staining was observed in the surrounding non-neoplastic tissue. Fifteen (14%) tumours were positive for *erb*B1, 19 (18%) for *erb*B2 and 44 (41%)

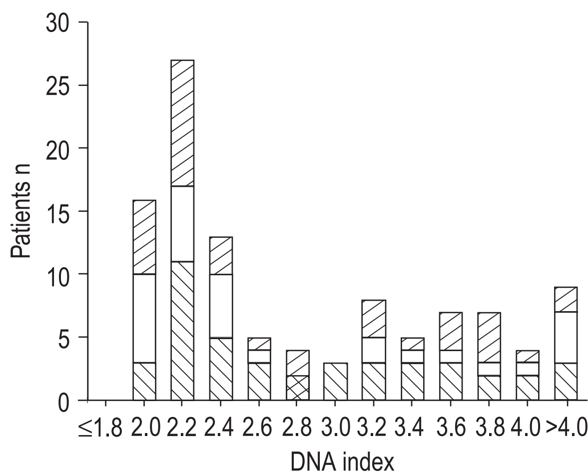

Fig. 1. – Distribution of deoxyribonucleic acid (DNA) indices (n=108; median 2.4; SD 0.79). ▨: squamous cell cancer; □: adenocarcinoma; ▧: large cell carcinoma.

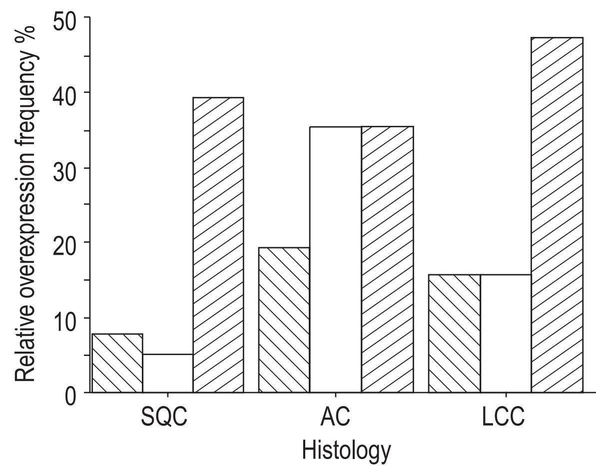

Fig. 2. – Relationship between expression of *erb*B1 (▧), *erb*B2 (□) and P53 (▨) and histological type (n=108; 107 for P53). SQC: squamous cell cancer (n=39; 38 for P53); AC: adenocarcinoma (n=31); LCC: large cell carcinoma (n=38).

for P53. Adenocarcinomas showed a significantly higher frequency of *erb*B2 overexpression (11 cases, 35%) in comparison to other histological subgroups (p=0.004). No other significant differences of gene expression were seen with regards to tumour stage or histology (fig. 2). No significant correlations were noted between *erb*B1, *erb*B2 and P53 expression or ploidy status.

After extraction of DNA, 53 samples showed sufficient DNA concentration and purity (fig. 3). After ddPCR, the gene dosage of *erb*B1 could be evaluated in 37 tumours with acceptable results from the negative control, the reference gene and the tumour sample itself (fig. 4): for *erb*B1, normal mean gene copy numbers (AGCNs) were found in 32 (86%) cases. Five tumours had an AGCNs of >1.6, *i.e.* three squamous cell carcinomas with AGCNs of <2.0 and two undifferentiated tumours with AGCNs of 2.4 and 4.3. All five tumours with AGCNs of *erb*B1 of >1.6 also showed overexpression. Fifty-two tumour samples were suitable for *erb*B2 analysis, of which 49

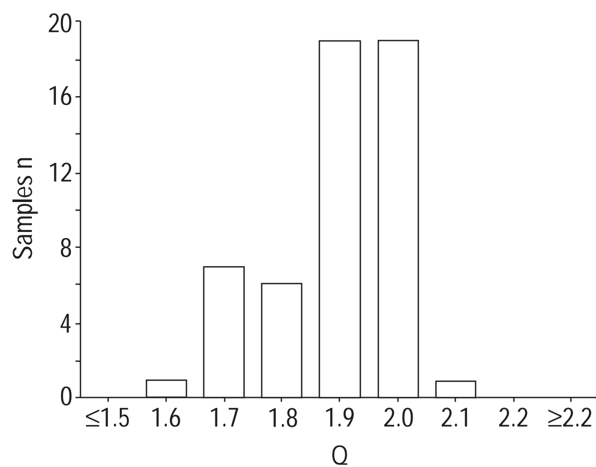

Fig. 3. – Degrees of purity of 53 tumour samples used for double-differential polymerase chain reaction after deoxyribonucleic acid isolation. Q: quotient of the corrected optical densities (ODs) measured photometrically at 260, 280 and 320 nm ($Q=(OD_{260}-OD_{320})/(OD_{280}-OD_{320})$).

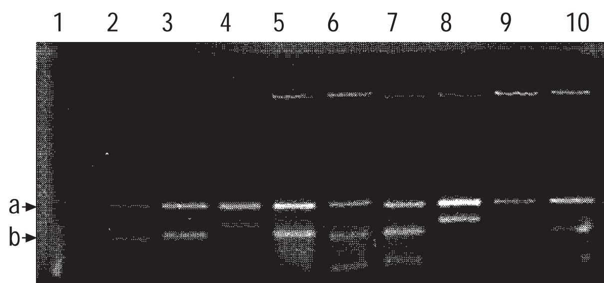

Fig. 4. – Electrophoretic analysis of the polymerase chain reaction (PCR) results obtained from three different non-small cell lung tumours. The degree of amplification was determined by comparison of the band intensity of the oncogene PCR product (b) with that of the reference gene band (a) in the ScanPack program (Biometra, Göttingen, Germany). Lanes 1–4; *erb*B1, *erb*B2, *erb*B3 and superoxide dismutase 2 (SOD2) (samples from tumour 1); lanes 5–8: *erb*B1, *erb*B2, *erb*B3 and SOD2 (tumour 2); lanes 9 and 10: *erb*B1 and *erb*B2 (tumour 3). No significant amplification could be detected. The density of the first lane could barely be assessed and was therefore excluded from further analysis.

tumours had normal AGCNs of *erb*B2. In addition, one adenocarcinoma had an AGCN of 2.0 and showed over-expression of *erb*B2. Two undifferentiated large cell cancers had AGCNs of 2.6 (without showing over-expression of *erb*B2) and 2.7. Expression of *erb*B2 in the latter tumour sample could not be determined because of repeated poor staining quality. Thus, interestingly, all tumours with more elevated *erb*B1 and *erb*B2 AGCNs were undifferentiated large cell carcinomas.

In 53 tumour samples, the AGCN of *erb*B3 could be determined. Thereby 51 (96%) tumours had a normal AGCN. Two tumours had slightly elevated AGCNs, *i.e.* one adenocarcinoma and one undifferentiated tumour with AGCNs of 1.7 and 1.8.

The mean follow-up was ~2 yrs (range 40–1,187 days, one patient with postoperative death on day 40). Fourteen patients were excluded from further analysis: in six patients, histological examination of the resected tissue revealed R1 resection, five died because of insufficiency of the bronchial stump or pneumonia within 30 days postoperatively and three were lost to follow-up. The patients with R1 resection underwent adjuvant local radiation of the tumor site with 54 Gy. Performing the Cox regression test, patients with R1 resection had a significantly worse prognosis ($p<0.0001$).

Ninety-seven patients with analysis of the cell cycle and 100 patients with known levels of *erb*B1, *erb*B2 and P53 expression were suitable for survival analysis. Using Kaplan-Meier estimates for univariate analyses, neither ploidy status, ploidy index nor number of stem lines were statistically significant prognostic parameters. Likewise, expression of *erb*B1, *erb*B2 and P53 was of no prognostic value. When a Cox regression model was used to analyse sex, tumour stage, histology, operative procedure, expression of *erb*B1, *erb*B2 and P53, ploidy status and ploidy index, only the tumour stage was of prognostic value ($p=0.0206$) for predicting both survival and the recurrence-free interval.

Discussion

In the present study, the prognostic value of ploidy status, expression of *erb*B1, *erb*B2 and P53, and gene dosage of *erb*B1, *erb*B2 and *erb*B3 were investigated prospectively. Patients with NSCLC (118) were included in the study. Patients with R1 resection followed by adjuvant radiation of the tumour site had a significantly worse prognosis ($p<0.0001$), which is in accordance with other reports [10]. With a mean follow-up of ~2 yrs only tumour stage was shown to be prognostically significant. No prognostic value of sex, histology, ploidy status, ploidy index, or expression of *erb*B1, *erb*B2 or P53 was observed. Ploidy was independent of outcome, histology, stage, grade of differentiation, and expression of *erb*B1, *erb*B2 or P53.

The reported results concerning DNA analysis by flow cytometry are inconsistent. There are no specific guidelines concerning interpretation of the histograms. Different diploid reference populations, use of paraffin-embedded material and different classification of tumours as euploid or aneuploid prevent comparisons [11]. Stromal cells are seen as the optimal reference population [11]. By using anticytokeratin as a second stain for epithelial cells, the sensitivity of the interpretation of the histograms is markedly improved [11, 12]. Most studies report an 80% prevalence of aneuploid tumours, which is in accordance with the present results [13, 14]. Occurrence of aneuploidy seems to be independent of histology or grading or stage of tumour [14–16]. Several studies denied a prognostic value of ploidy [14, 16]. MÖRKVE *et al.* [16] analysed ploidy and the expression status of P53 in 112 NSCLCs using flow cytometry. As in the present study, ploidy was not correlated to expression of P53 or prognosis [16]. By using flow cytometry without second staining, differentiation between malignant and benign cells is not possible and staining artefacts cannot be excluded [11]. Therefore, their results concerning P53 expression cannot be compared with the present ones [16].

In contradistinction to breast cancer studies, there are only few studies concerning amplification of the *erb*B family in NSCLC [17, 18]. Including the present series, it seems that amplification of the *erb*B family is a rise event in NSCLC and a rare reason for overexpression. Causes other than amplification are known to give rise to over-expression and may be the reason for these results [2]. SHIRAISHI *et al.* [17] reported on 10 NSCLCs with amplification of *erb*B1 of 114 tumours. Only one tumour of 51 investigated samples had an amplified *erb*B2 gene. SLEBOS *et al.* [18] failed to detect any amplification of *erb*B1 or *erb*B2 in 43 NSCLCs by Southern blot analysis.

A study performed by GORGOULIS *et al.* [19] examined the protein expression of paraffinized material from 40 squamous cell cancers immunohistochemically and investigated possible gene amplification using the semiquantitative differential PCR technique. Although over-expression was present in 65% for *erb*B1, 28% for *erb*B2 and 10% for *erb*B3, *erb*B1 amplification was detected in only 11 (28%) cases. There was only one tumour with amplified *erb*B2 and no tumours with amplification of *erb*B3 [19]. Similar to the present study, all tumours with *erb*B1 amplification showed over-expression. A tumour was defined as overexpressing a gene when either the cytoplasm or cell membranes were stained. There were no tumours showing overexpression of all investigated genes [19]. Because of consideration of the staining of both cytoplasm and cell membranes, the occurrence of overexpression may have been

overestimated. Furthermore, any information about a necessary percentage of positive cells for classification as overexpressing a gene is missing [19].

The level of expression of *erb*B1 and *erb*B2 described in the literature varies considerably. The diversity of the results is influenced by various factors such as the ability of the antibody used to detect overexpression [20, 21] and the threshold value for assessment of positivity [20, 22, 23]. The threshold value for assessing positivity used by other groups has ranged from several positive cells to >80% positive [20]. In the present study, only membrane staining was considered and a threshold value of 5% used in order not to underestimate overexpression of *erb*B1. However, the clear majority of the present samples showed staining of markedly >5% of the tumour cells.

In the present study, expression of *erb*B1 and *erb*B2 was investigated immunohistochemically in 108 tumours in paraffin blocks. Although overexpression of *erb*B2 was found in 18% of the tumours, which is consistent with the results reported to data by other groups [2, 23], the results showed an occurrence of expression of *erb*B1 of 14%. Adenocarcinomas showed significantly higher expression of *erb*B2. There were no significant correlations between tumour stage, expression of *erb*B1, *erb*B2 or P53, or ploidy status.

The lower frequency of *erb*B1 expression compared to the literature [20, 22, 23] could have several explanations. It might be the result of more selective criteria of assessment of positivity (membrane staining only). However, it cannot be excluded that the sensitivity of detection of *erb*B1 expression may have been decreased by the fixation and paraffin embedding procedure [24].

PFEIFFER *et al.* [22] investigated the immunohistochemical expression of *erb*B1 and *erb*B2 in cryosections of 186 NSCLCs. Expression of *erb*B1 was higher in squamous cell carcinomas, whereas the level of *erb*B2 staining was higher in adenocarcinomas. Expression of either or both receptors was not correlated with prognosis [22].

Recently, PASTORINO *et al.* [23] published a retrospective study analysing the expression of *erb*B1, *erb*B2 and P53 in 515 cases of pathological stage I NSCLC immunohistochemically. None of these markers emerged as an independent predictive factor in survival.

P53 has been identified as a product of a tumour suppressor gene which is frequently mutated in common human cancers including NSCLC [25]. Using a threshold value of 5% for the reasons indicated above, the prevalence of P53 overexpression was 41% in the present study, which is similar to the results of other studies [26, 27]. Several studies denied a prognostic value of P53 accumulation [4, 23, 25, 28, 29]. MCLAREN *et al.* [29] investigated 125 primary lung tumours using a panel of five anti-P53 antibodies and could not show any differences in survival between P53-positive and -negative cases. The polyclonal antibody CM-1, which was also used in the present study, produced quite intense staining compared to other markers [29]. These results indicate that, although P53 may be of considerable importance in the initiation of malignancy, it is probably of little prognostic significance once a tumour has developed [29].

The present data confirm the results of several previous studies that demonstrated no prognostic value of ploidy and expression of *erb*B1, *erb*B2 and P53. Amplification of the *erb*B family seems to be a rare event and not the cause of overexpression in most non-small cell lung cancers. In addition, P53 accumulation does not seem to be an indicator of worse prognosis. However, the purpose of using *erb*B1, *erb*B2 or P53 as targets in anticancer therapy needs to be determined [4]. Further studies are necessary to obtain a more precise idea of the molecular changes and pathobiological pathways that occur when lung cancer develops. Hopefully, with a better understanding of cancer genetics, new prognostic markers can be determined in order to better develop new therapeutic strategies.

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