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Overexpression of c-erbB2 protein correlates with disease-stage and chromosomal gain at the *c-erbB2* locus in non-small cell lung cancer

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Abstract

Overexpression of the c-erbB2 protein is observed in a variety of malignancies including non-small cell lung cancer (NSCLC). We aimed to determine the rate of *c-erbB2*-overexpression in our tumour collection and to clarify its correlation with the chromosomal status at the c-erbB2 locus 17q21 in NSCLC. Eighty-nine NSCLC were analysed immunohistochemically using a polyclonal c-erbB2 antibody (DAKO). The staining was scored according to the guidelines of the Clinical Trial Assay recommendations (0–3+). Of these, 44 cases were also analysed by comparative genomic hybridisation (CGH). Overexpression was observed in 37% of the cases (score > 1) which was associated with higher disease stages and a positive nodal status in adenocarcinomas. Chromosomal gains at 17q21 were clearly correlated with overexpression of the gene ($P = 0.009$). In addition, there was a highly significant correlation between the c-erbB2 expression comparing the whole section immunostaining analysis and a 127 lung tumour tissue array which included 74 of the 89 cases that were analysed by the classical procedure. We conclude that c-erbB2 is a marker of tumour progression in NSCLC which can be observed on protein level and reflects chromosomal alterations at 17q21.

Keywords: Non-small cell lung cancer; c-erbB2; Comparative genomic hybridisation; Immunohistochemistry; Tissue array

1. Introduction

Lung cancer is a major cause of death from neoplastic malignancy in the western world. In the USA in 1998 alone, 172 000 new cases were diagnosed, while 160 000 patients died of the disease [1]. The mortality rate of lung cancer is amongst the highest of all cancer types. In the last 20 years, the mortality rate was lowered by only 6% as a result of improved therapies. So far, the most promising approach appears to be primary and secondary prevention, as shown in recent studies [2].

A well established screening method to detect the genetic changes that underly carcinogenesis is comparative genomic hybridisation (CGH) first introduced by Kallioniemi and colleagues in 1992 [3]. It allows the localisation and identification of chromosomal aberrations defined as chromosomal gains or losses. Non-small cell lung carcinoma (NSCLC) are characterised by a quite complex pattern of changes. Deletions are commonly found in the regions 1p, 1q, 2q, 5q, 6p, 8p, 8q, 10q, 11p, 11q, 14q, 17q, 18q and 22q, while chromosomal overrepresentations occur at 1p, 1q, 2p, 3q, 5p, 8q, 11q, 16p, 17q and 19q [4,5].

For some of the chromosomal gains, the amplification of the underlying oncogene can be identified such as *c-myc* at 8q24 and *cyclin D1* at 11q13 [33,34]. However, for the majority of loci, the associated genetic defects are still unknown.

In former CGH based studies, we found frequent chromosomal overrepresentations of chromosome 17q harbouring the locus of the *c-erbB2* gene at 17q21 in NSCLC. The oncoprotein c-erbB-2, a 185kd protein with tyrosine kinase activity belonging to the epidermal growth factor receptor (EGFR)-family was shown to be involved in various human malignancies [6]. In NSCLC, the most common form of lung cancer, c-erbB2 overexpression is commonly observed, although to a variable extent [7–18]. The importance of c-erbB2 overexpression for the prognosis in NSCLC is still controversial [14–17].

The aim of this study was to evaluate the status of c-erbB2 expresssion in our set of patients and to

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investigate a possible association of c-erbB2-protein expression determined by immunohistochemistry with the chromosomal status at the *c-erbB2* locus 17q21. Furthermore, c-erbB2 expression was correlated with clinicopathological parameters and disease stage according to The International Union Against Cancer (UICC).

2. Patients and methods

2.1. Tumour samples

Our sample consisted of 89 patients between the ages of 42 and 80 years (mean 63 years) who underwent thoracotomy for resection of NSCLC in the Department of Surgery of Charité University Hospital from 1995 to 1997. Of these, 44 cases were analysed by CGH. No adjuvant radiotherapy or chemotherapy was administered before surgery. Table 1 summarises the clinicopathological characteristics according to TNM criteria of the UICC [19].

Tumour specimens were transferred to the Institute of Pathology within 1 h after surgical removal. For CGH analysis, DNA was extracted from several 30 μm cryostat tissue sections by proteinase K and phenol-chloroform extraction which was verified to consist of a minimum of 70% tumour cells in each case. The histopathological diagnosis was established in every case according to the World Health Organization (WHO) guidelines [20].

Table 1
Study cohort

	c-erbB2 low *n* (%)	c-erbB2 high *n* (%)
Total no. ($n=89$)	56 (63)	33 (37)
Adenocarcinoma	29 (33)	17 (19)
SCC	27 (30)	16 (18)
Grade 1/Grade 2[a]	33 (37)	13 (15)
Grade 3	23 (26)	20 (22)
pT1	15 (17)	6 (7)
pT2 + [b]	41 (46)	27 (30)
pN0	36 (40)	14 (16)
pN1 + [c]	20 (22)	19 (21)
Stage I–IIa	36 (40)	12 (13)
Stage IIb–IV	20 (22)	21 (24)
CGH cases ($n=44$)	29 (66)	15 (34)
17q21 status (99% CI)		
Gain	13 (30)	12 (27)
No gains	16 (36)	3 (7)
17q21 status (0.8/1.2)		
Gain	7 (16)	10 (23)
No gain	22 (50)	5 (11)

SCC, squamous cell carcinoma; CGH, comparative genomic hybridisation; CI, confidence interval.

[a] Grade 1 ($n=3$), Grade 2 ($n=30$).

[b] pT2 ($n=63$), pT3 ($n=3$), pT4 ($n=2$).

[c] pN1 ($n=11$), pN2 ($n=29$).

2.2. Tissue array generation

Suitable areas for tissue retrieval were marked on standard haematoxylineosin (HE) sections, punched out of the paraffin block and inserted into a recipient block as described in Ref. [41]. The tissue arrayer was purchased from Beecher Instruments (Woodland, USA). The punch diameter was 0.6 mm. The lung tumour array used for immunohistochemistry consisted of 150 tissue samples of which 127 were NSCLC. Amongst these were 74 cases of the 89 cases used for the whole section immunohistochemical analysis. The tissue array was cut in 4 μm sections without any sectioning aids like adhesive tapes or additionally coated slides. The immunostaining protocols were identical.

2.3. Comparative genomic hybridisation

CGH was performed as previously described [5]. Protocols for the preparative steps are available at our web site (http://amba.charite.de/cgh). Similarly, digital image analysis was done as previously reported [22]. Generally, 15 metaphases/karyograms were analysed for each case. CGH sum karyograms as well as mean ratio profiles with confidence intervals (CI) were calculated [21]. Alterations were determined by calculating the mean FITC/TRITC (ratio of fluorescence images of the test genome/reference genome) profile (i.e. the ratio of chromosomal losses/gains) with its 95 and 99% CI. The profile was tested for significant deviations from the normal ratio of 1.0 by applying a Student *t*-test, as well as using fixed ratio thresholds (0.8/1.2) [21,22]. Whereas the fixed thresholds represent a rather stringent criterion for the determination of DNA imbalances, the statistical method using the *t*-test is a quite sensitive measure.

2.4. Immunohistochemistry

Formalin-fixed paraffin-embedded archive material was retrieved from the files of the Institute of Pathology of the Charité University Hospital. The blocks were freshly cut (4 μm), sections were mounted on superfrost slides (Menzel-Gläser, Germany) and dewaxed with xylene and gradually hydrated. Antigen retrieval was achieved by pressure cooking in 0.01 M citrate buffer for 6 min. The primary antibody was a polyclonal c-erbB2 antibody (DAKO, Germany) which was diluted 1:75 using a background reducing dilution buffer from DAKO. No other blocking agents were employed. The primary antibody was incubated at room temperature for 1 h. Detection took place by the conventional labelled-streptavidin-biotin (LSAB-kit, DAKO) method with alkaline phosphatase as the reporting enzyme according to the manufacturer's instructions. Fast-Red (Sigma-

chemicals) served as the chromogen, afterwards the slides were briefly counterstained with haematoxylin and aquaeously mounted.

For evaluation of the c-erbB2 staining, the Clinical Trial Assay (CTA) criteria were employed, which are also the basis for the commercial Hercept-test (DAKO). The slides were independently examined by two clinical pathologists.

2.5. Statistical analysis

Fisher's exact test which is especially suited for small sets of data was used to determine the strength of association between the investigated parameters. To compare the expression of c-erbB2 with clinicopathological parameters, 2×2 contingency tables (e.g. c-erbB2 score 0–1 versus 2–3 and G1&G2 versus G3) were set up and the resultant P value was calculated. P values $\leqslant 0.05$ were considered significant. All calculations were performed on a PC using the statistical software package NCSS.

3. Results

3.1. Immunohistochemistry

44 cases (50%) exhibited no relevant c-erbB2 staining (Fig. 1a). In 12 cases (13%), a minimal circumferential membranous staining of more than 10% of the tumour cells was observed (Fig. 1b). 20 cases (22%) showed a moderate staining intensity (Fig. 1c), 13 cases (15%) had a strong staining signal (Fig. 1d). Taking groups three and four together 37% of tumours showed an overexpression of c-erbB2 in this study (Table 1).

No correlation between c-erbB2 expression and most single clinicopathological parameters (sex, age, tumour grade, size) could be demonstrated. Only a positive nodal status was significantly correlated to c-erbB2 overexpression ($P = 0.05$). Stratifying the data, we repeated this analysis for squamous cell carcinomas (SCC) and adenocarcinomas, respectively. Here a significant association of a positive nodal status and c-erbB2 overexpression ($P = 0.016$) was seen for adenocarcinomas, whereas squamous cell carcinomas showed no correlation at all ($P = 1$ (Table 2)).

To determine if c-erbB 2 expression was associated with disease stage (UICC), the cases were sorted according to the stage of disease and split in two groups. One group comprised stages I–IIa, the other stages IIb–IIIb. Fisher's exact test revealed a P value of 0.015, thus demonstrating a significant association of high c-erbB2 scores (>1) with advanced disease stages ($>$ IIa).

3.2. Correlation between whole mount section and lung tumour tissue array analysis

Comparing the immunohistochemical evaluation of the standard slides with the tissue array slides, we found

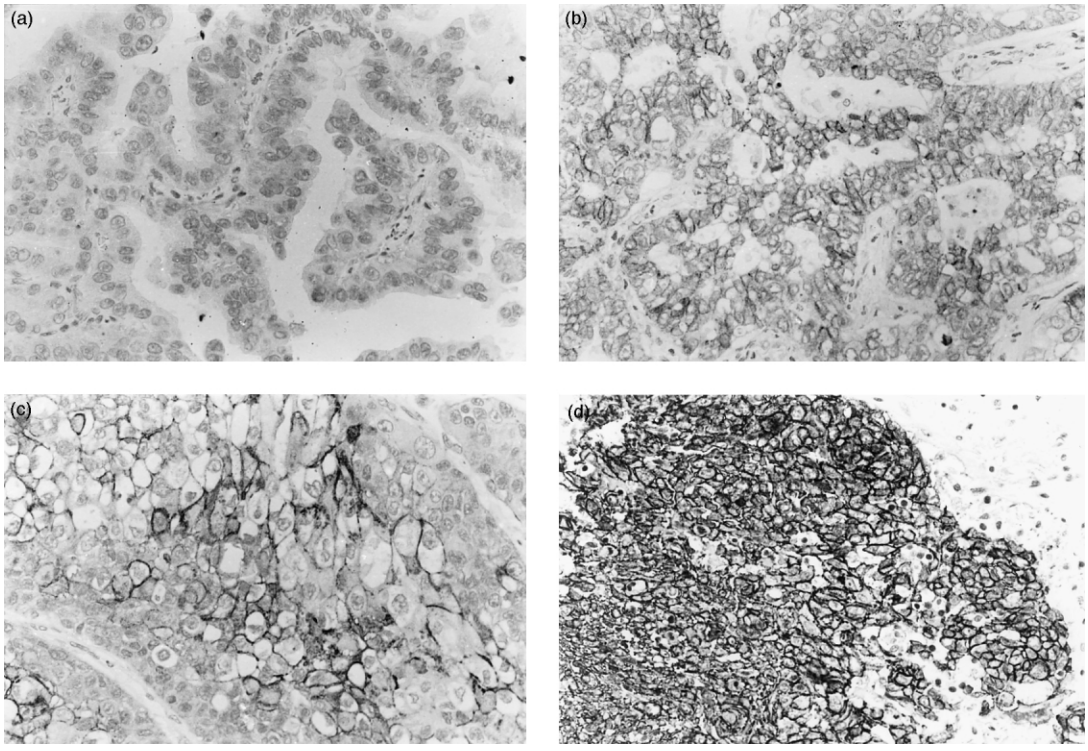

Fig. 1. Examples of the immunohistochemical assessment of c-erbB2 in non-small cell lung carcinoma (NSCLC) by the DAKO polyclonal antibody illustrating the Clinical Trial Assay (CTA) scoring system. (a) no or minimal staining, (b) weak membranous circumferential expression (1+) in at least 10% or more of the tumour cells, (c) and (d) moderate (2+) and strong staining (3+), respectively.

Table 2
Correlations between c-erbB2 overexpression and clinical/genetic data (P values[a])

	Stage (UICC)	Grading	pN+	pT	Gain at 17q21
SCC	0.752	0.537	1	0.443	0.077
ADC	0.013	0.126	0.016	0.09	0.146
All tumours	0.015	0.084	0.05	0.443	0.009

SCC, squamous cell carcinoma; ADC, adenocarcinoma.
[a] According to Fisher's exact test.

a highly significant correlation of both methods (Fisher's exact test, $P<0.001$). Of the 74 overlapping tumour cases, 41 (55%) showed no relevant c-erbB2 expression and 17 (23%) exhibited a c-erbB2 overexpression in both methods. 12 cases (16%) were conventionally diagnosed as overexpressers without or with only weak staining in the array, and 4 cases (5%) had a reversed mismatch.

3.3. Comparative genomic hybridisation

An unselected subset of 44 NSCLC was analysed by CGH. The histogram of the chromosomal imbalances is shown in Fig. 2. It represents the DNA gains and losses as incidence curves along each chromosome [10]. Applying two different criteria commonly used for determining chromosomal imbalances by CGH, i.e. Student t-test with 99% confidence and 0.8/1.2 fixed ratio thresholds, we observed chromosomal gains at 17q21 in 25 (57%) and 17 (39%) of the 44 cases, respectively. In Fig. 3, the histogram illustrating the incidence of chromosomal alterations using the fixed ratio thresholds for chromosome 17 is shown. The *c-erbB2* locus is indicated by a horizontal bar. There was a significant association of the immunohistologically determined *c-erbB2* overexpression with the chromosomal gain at the c-erbB2 locus 17q21 as detected by CGH. This association was strongest when the fixed FITC/TRITC ratio thresholds 0.8/1.2 were used ($P=0.009$), but also reached significance using the more sensitive statistical method ($P=0.05$).

4. Discussion

In this study, NSCLC were immunohistologically examined for the expression of c-erbB2 and additionally characterised by CGH.

The results demonstrate a high incidence of c-erbB2 overexpression (37%) in NSCLC. This is, in general, in concordance with other researchers whose results are briefly summarised in Table 3. The different rates of

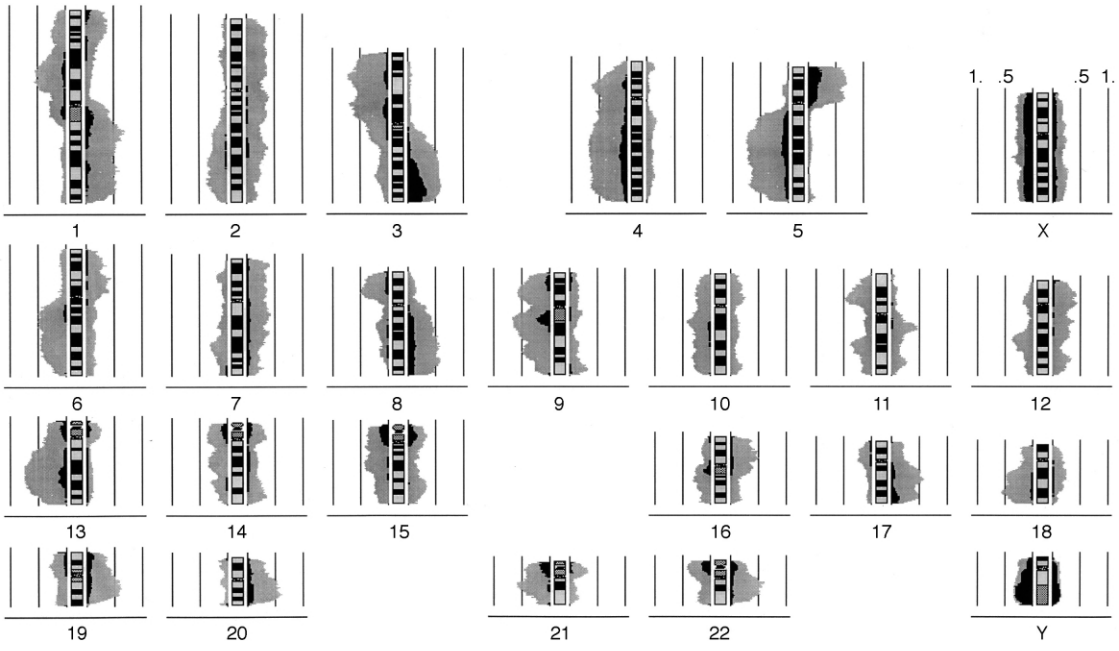

Fig. 2. Histogram of chromosomal imbalances in 44 NSCLC using a statistical test for the determination of CGH results (Student t-test, 99% significance, threshold 1.0/1.0). Deletions are shown on the left side of the ideograms and overrepresentations on the right side. The vertical lines symbolize the 0, 50 and 100% incidence, respectively, e.g. the maximum value of 100% is reached if all tumours of the tumour group carry a change at a specific chromosomal region. The proportion of pronounced DNA gains and losses being defined as imbalances for which the ratio profiles exceeded the thresholds of 1.5 and 0.5, respectively, are visualised in black. They are most likely to represent high copy amplifications or multi-copy deletions. Since the copy number of an amplification can not be determined by CGH, the distinction between normal and pronounced DNA gains represents one possibility of a semiquantitative measure of a DNA overrepresentation. For the locus of the *c-erbB2* gene at chromosome 17q21, DNA overrepresentation were observed in 57% of the cases ($n=25$) of which 2 had pronounced DNA gains.

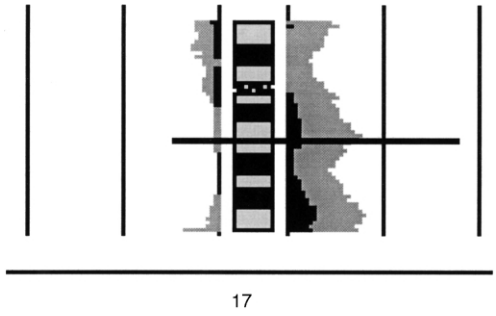

Fig. 3. Histogram of chromosome 17 using the fixed ratio thresholds 0.8 and 1.2 (and no statistics) for the determination of deletions and overrepresentations, respectively. These criteria are more selective than the statistical method of which the result is shown in Fig. 2. The *c-erbB2* locus at 17q21 is indicated by the black horizontal line. Of the 44 cases, 17 harboured a DNA gain including the 2 cases with the pronounced change. The association between overexpression of the gene (score 2+ and 3+) and DNA overrepresentation was highly significant ($P=0.009$).

c-erbB2 overexpression published so far can be attributed to varying tumour sets and also to methodological differences (antibody, detection system) and probably most importantly to different approaches in the interpretation of the staining. As c-erbB2 commonly shows a cytoplasmic, as well as more or less pronounced membranous staining pattern, both have been used by various researchers for correlations with clinicopathological data leading to different results. Giatromanolaki and colleagues found the membranous staining impossible to evaluate because of an invariable cytoplasmic background staining which they preferably subjected to statistical analysis [13,14]. No association of c-erbB2 expression with clinicopathological data could be demonstrated.

Pastorino and colleagues were more selective in assessing the c-erbB2 status and utilised the membranous staining only. This resulted in the lowest c-erbB2-expression published so far in NSCLC (4%), again with no correlation to clinicopathological parameters in their tumour set which was restricted to stage I lung cancers [16].

An earlier study by Shi and colleagues graded c-erbB2 expression from absent to strong (0, +, ++, +++), but apparently used any expression for positivity [10]. They found a significant association of higher c-erbB2 expression with an advanced disease stage. Furthermore, they reported a significantly more frequent c-erbB2-expression in node-positive SCC than negative ones.

In this study, we used a polyclonal c-erbB2-antibody (DAKO) which is widely used in gynecopathology. We applied the scoring recommendation of the CTA to the evaluation of c-erbB2 expression in NSCLC: only circumferential membranous staining in at least 10% or more of the tumour cells was regarded positive and semi-quantitatively scored (1+, 2+, 3+). Only moderate and strong stainings (2+ and 3+) were considered as an indicator of c-erbB2 overexpression.

Our data shows a significant association of c-erbB2 overexpressing tumours with a higher clinical stage (>IIa according to UICC). This endorses the findings of Shi and colleagues [10]. They had reported an overexpression of c-erbB2 in 81 and 87% of stage II/III cases, while stage I cases had an overexpression rate of 50%. Tateishi and colleagues [8] had also noted a higher c-erbB2 expression in advanced disease stages. Other researchers found no correlation for c-erbB2 expression with disease stage [9,15,17,18].

In our tumour set, the rates of c-erbB2 expression for adenocarcinomas and SCC are similar with 36.9 and 37.2%, respectively. This contrasts with the data already published: Tateishi and colleagues [8] found an overexpression in 28% of adenocarcinoma and 2% of SCC. Pastorino and colleagues published 6% for ADC and 2% for SCC [16]. Pfeiffer and colleagues demonstrated a high expression of c-erbB2 in 30% of ADC and 14% for SCC [15]. Using tumour lysates, Lopez-

Table 3
Studies on c-erbB2 expression in NSCLC

Author [Ref.]	Year	c-erbB2-(Over)expression (%)	Type of study	No. of cases
Kern [7]	1990	33	IHC, paraffin	55
Tateishi [8]	1991	17	IHC, paraffin	203
Volm [9]	1992	35	IHC, paraffin	81
Shi [10]	1992	59	IHC, paraffin	120
Bongiorno [11]	1994	93	IHC, cryosections	29
Harpole [12]	1995	13	IHC, paraffin	150
Giatromanolaki [13,14]	1996	(19), 37	IHC, paraffin	107
Pfeiffer [15]	1996	26	IHC, cryosections	186
Pastorino [16]	1997	4	IHC, paraffin	608
Hsieh [17]	1998	50	IHC, paraffin	42
Lopez-Guerrero [18]	1999	22	Quantitative ELISA, fresh tissue	64

IHC, immunohistochemistry; NSCLC, non small cell lung carcinoma; ELISA, enzyme-linked immunosorbent assay.

Guerrero and colleagues [18] found a higher c-erbB2 content in ADC. We can only speculate on the high rate of c-erbB2 expression that we found in SCC. Still, our data revealed significant differences in both histological subtypes for the correlation of c-erbB2 overexpression was clinical stage, nodal status and tumour size. The stage association of c-erbB2 expression was highly significant in ADC ($P=0.013$), whereas in SCC ($P=0.752$) no relevant association was found. That this stage dependency was still significant for all tumours (ADC + SCC; $P=0.015$) might be attributed to the slightly higher number of ADC cases (52%). The same phenomenon is reflected and probably partly the basis of the association of c-erbB2 overexpression and the nodal status. Again, a significant ($P=0.016$) association was found for ADCs alone, but not for SCC ($P=1$). Shi and colleagues [10] reported a positive correlation of c-erbB2 expression and positive nodal status in SCC alone. We can not explain this discrepancy; most likely it may be due to general differences in the methods, as well as in the tumour set used.

There was an excellent concordance of the results of immunohistochemistry of conventional slides compared with the arrayed tumour tissue. This must be attributed to a fairly even distribution of c-erbB2 expression in the lung tumours. Still, differences became apparent in a subset which is due to the small tissue sample size represented in the tumour punch. Although the results encourage the use of tissue arrays in research it also points to the need to test the staining of individual antigens in whole mount tissue sections prior to the uncritical usage of this new technology.

To our knowledge, this is the first study to link c-erbB2 protein overexpression to chromosomal gains at the *c-erbB2* locus in NSCLC. Similar to the semiquantitative assessment of gene expression by immunohistochemistry there is a certain variability in the interpretation of CGH results. Since the associations were significant using both a rather sensitive, as well as a more stringent, method for the determination of chromosomal imbalances, we are convinced that this is a valid finding. The frequency of 39% DNA overrepresentation at chromosome 17q21 using the fixed ratio thresholds was very close to the 37% of cases with CTA scores 2+ or 3+ and there was the highest significance using the fixed ratio thresholds.

In general, our CGH data in NSCLC [5,31] being the largest collection studied so far correlate with that of other researchers [35–38]. Two studies reported gains at 17q in NSCLC [35,36], whereas two others did not [37,38]. In the latter, rather stringent criteria were used for the determination of DNA imbalances and therefore gains were probably missed. Consistent with this observations, we found only a very small percentage of cases with a pronounced gain pointing to high copy amplifications. However, it is important to note that the overrepresentation at 17q is well localised and can not be attributed to polysomy of chromosome 17 or the entire long arm. This observation is also supported by classical cytogenetics [39,40].

In breast cancer, the correlation between the c-erbB2 expression and the genetic status has already been well established [30]. Recently, the analysis of this gene has reached considerable clinical importance due to the availability of a humanised c-erbB2 antibody that is now being used for the treatment of patients in an advanced stage of the disease [23–27]. However, a prerequisite for the application of this new therapeutic option is the determination of the overexpression and/or amplification of the gene. Genetic analysis is still regarded as the gold standard since amplications of the gene were initially found to be correlated with tumour progression [29]. Technically, however, the immunohistochemical analysis is much faster and thus the method of choice for a routine analysis. We feel that it is important to note that simple immunostaining can replace a laborious genetic analysis to assess the chromosomal status at a specific locus. This is even more important since CGH has already highlighted many candidate regions of which the gain or loss has an impact on progression and prognosis [28,31,32].

In summary, our findings endorse the hypothesis that c-erbB2 upregulation is associated with tumour progression in lung cancer. However, there are no simple relationships with the tumour phenotype since overexpression does not correlate with all clinicopathological parameters of progression and the correlations seem to be particularly strong for certain tumour subgroups. Thus, the role of c-erbB2 in lung cancer biology clearly necessitates further studies especially given the prospect of new therapy with the humanised monoclonal antibody.

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