EGFR/erB-1, HER2/erB-2, CK7 and LP34 expression in preneoplastic lesions of respiratory epithelium: an immunohistochemical and genetic study

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ABSTRACT

An interpretative diagnosis of preneoplastic lesions would have impact in lung cancer early diagnosis and survival together with the study of Erb-B family receptors as they have an important role in lung carcinogenesis. The existence of tirosine kinase inhibitors stresses the
importance of studying gene alterations with possible defined implications in patients’ selection for chemoprevention schemes and characterization of carcinogenesis.

This study concerns bronchial preneoplastic lesions observed in biopsies as basal cell hyperplasia, squamous metaplasia and dysplasia. Using immunohistochemistry, those lesions were characterized by antibodies applied against LP34 (high weigh molecular cytokeratin), CK7, Chromogranin A, Ki67, p53, C-erbB-2 and EGFR. HER2 and EGFR gene copy number was also evaluated by fluorescent in situ hybridization (FISH) in those lesions.

The expected results defined the origin cell for adaptative and preneoplastic lesions. By known experiences and published data, beyond the stem cell, the spectral evolution of bronchial preneoplastic lesions was demonstrated by characterizing basal cells (LP34) and their neoplastic potentiality.

Dysplasias showed a higher expression of EGFR, Ki67 and p53 with a stepwise increase with the gravity of the preneoplastic lesions. C-erbB-2 immunohistochemical overexpression is a rare event in preneoplastic lesions. Polyssomy was the main mechanism for EGFR and HER2/neu high gene copy number.

HER2 and EGFR will be expected to understand early carcinogenesis as well as increased proliferation index (Ki67).

**Key Words:** bronchial epidermoid metaplasia; EGFR, HER2/c-erbB-2; CK7; LP34.

*Acknowledgments:* Isabel Carreira; Paula António.
INTRODUCTION

Lung cancer is the leading cause of death by malignancy in developed countries and throughout the world. Environmental and occupational exposures, such as polyaromatic hydrocarbons, radon, asbestos, nickel, arsenic and chromium are important determinants of lung cancer risk, but cigarette smoking is the main risk factor, accounting for about 90% of the cases in men, 70% of the cases in women and with in today ex-smokers comprising nearly 50% of all new lung cancer cases (13,59,37,1,65).

The overall 5-year survival rate for lung cancer patients remains less than 15% (41) and the death rate for lung cancer exceeds the combined total for breast, prostate and colon cancer in developed countries (65). The major reasons for the poor prognosis for lung cancer are the lack of effective screening and early diagnosis procedures, the propensity for early metastasis and the inability of systemic therapies to cure patients with widely metastatic disease (21). Lung cancer is the result of a multi-step accumulation of genetic and/or epigenetic alterations, therefore a better understanding of the molecular mechanism by which these alterations affect lung cancer pathogenesis would provide new and more effective strategies for chemoprevention, early diagnosis and targeted treatment (58). The existence of novel drugs against specific molecular targets like EGFR and HER2/neu genes leads the importance of defining their expression as biomarkers in preneoplastic lesions.

The WHO lung tumour classification recognises three types of preinvasive lesions: squamous dysplasia and carcinoma in situ (CIS); atypical adenomatous hyperplasia (AAH) and diffuse idiopathic pulmonary neuroendocrine cell hyperplasia (DIPNECH) (64). Respiratory epithelium adaptative lesions include basal cell hyperplasia, squamous metaplasia, followed by dysplasia and CIS which precede or accompany microinvasive or
invasive carcinoma. Hyperplasia and squamous metaplasia are considered reversible lesions, while dysplasia and CIS are not (3,12,67,15,50,53).

Squamous metaplasia as a common adaptative lesion of the bronchial mucosae, especially in smokers, represents a precursor step in the development of invasive squamous cell carcinoma, deserving an accurate study to define it’s evolutionary potential to lung cancer, in order to be determined in bronchial biopsies (3,47,26,50).

Several molecular and genetic abnormalities have been described in lung carcinogenesis, including activation of proto-oncogenes and loss of tumour suppressor genes and DNA repair genes. Proto-oncogenes can be activated by point mutations, amplification, chromosomal rearrangement and overexpression. Tumour suppressor genes can be inactivated through the deletion of one parental allele combined with a loss-of-function mutation or aberrant methylation of a target tumour suppressor gene in the remaining allele (18, 14, 34). Those genomic and molecular abnormalities are early events in lung carcinogenesis and can persist in bronchial lesions for months or years and their persistence or regression have been correlated with the lesions’ evolution (44). Genetic and molecular alterations identified in preneoplastic lung lesions include loss of heterozygosity (LOH) at several loci (3p, 9p, 8p and 17p), Cyclin D1 overexpression, P53 mutations and increased immunoreactivity, MYC and RAS up-regulation, DNA aneuploidy and Bcl-2 overexpression. These alterations are responsible for the aberrant signal transduction (ST), cell cycle disregulation, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis and immune tolerance inherent in the lung malignant process (22,38,6,56,9,53). The earliest changes described in bronchial epithelium are LOH 3p, then 9p, 8p, 17p, 5q and RAS mutations (62,28,31,68). Molecular changes are recognized not only in lung cancer, but also in bronchial epithelium and parenchyma of current and former smokers without the development of lung cancer (66,17).
Epidermal growth factor receptor (EGFR; ErbB-1 gene) and ErbB-2 (HER2/neu) genes belong to ErbB family, a family of tyrosine kinase receptor proteins (TKs) that includes ErbB-3 (HER-3) and ErbB-4 (HER-4). The ErbB receptors are composed of three major domains: an extracellular for ligand binding; a transmembranar one and a cytoplasmic with tyrosine kinase activity. ErbB receptor function begins upon ligand binding and is followed by receptor homo (e.g., Erb-1/Erb-1) or heterodimerization (e.g., Erb-1/Erb-2), activating the tyrosine kinase domain of the receptor, which becomes phosphorylated and acts as a docking site for downstream signalling molecules and cytoplasmic messenger proteins, that then trigger a cascade of cytoplasmic and nuclear pathways (53).

It has been suggested and supported by experimental data that aberrant activation of the kinase activity of ErbB receptors plays an important role in the neoplastic transformation and progression, prognosis, survival and resistance to cytotoxic therapies (61,5,60,51,69,41,49,21,53). EGFR’s high expression is an early event in preneoplastic lesions, with an increasing expression from normal mucosae to hyperplasia, metaplasia and dysplasia (54,35,16,39,41,49). EGFR is overexpressed in 84% of squamous cell carcinoma, 65% of adenocarcinomas and 68% of large cell lung carcinomas (16). As it seems that ErbB2 is also highly expressed in bronchial preneoplastic lesions and its overexpression is less common in carcinomas (less than 35% of NSCLC, mainly adenocarcinomas) (18,16,23). The heterodimer EGFR/HER2-neu has been shown to have a stronger proliferative effect than the corresponding homodimers (4,48,20,49). Amplification and polyssomy of EGFR and HER2-neu and overexpressed proteins have been related to survival in patients with NSCLC (non-small-cell lung cancer), but contradictory results have been published (7,63,8,10,36). New therapies have also been developed recently directed to these two molecular targets, some of them being applied in chemoprevention trials stressing the necessity to understand their expression in adaptative and preneoplastic lesions (32,33, 65,27,43,55).
This study concerns bronchial preneoplastic lesions observed in biopsies as basal cell hyperplasia, squamous metaplasia and dysplasia. Using immunohistochemistry, those lesions were characterized by antibodies applied against LP34, CK7, Chromogranin A, Ki67, p53, C-erbB-2 and EGFR. HER2 and EGFR gene copy number was also evaluated by fluorescent in situ hybridization (FISH), using normal controls. The aim of the present study is to achieve a better understanding of the spectral evolution of bronchial adaptative and preneoplastic lesions, by recognizing the expression of EGFR and HER2-neu as a potential biomarker for lung cancer risk assessment. This knowledge would help to provide new and more effective strategies for chemoprevention, early diagnosis and targeted treatment.

**MATERIAL AND METHODS**

**MATERIAL**

A number of 67 bronchial biopsies were included in this study, comprising 89 preneoplastic lesions, selected from our database, from 2003 to 2007, considering the preservation, number and dimension of the small fragments obtained by bronchial fibroscopy. The population consisted in 57 men and 10 women, with 66.3 years as mean age (range 38-92), represented by 16 basal cell hyperplasia (BCH), 40 squamous metaplasia (SM) and 33 epidermoid dysplasia (7 mild dysplasia, 7 moderate dysplasia and 19 severe dysplasia/CIS) were identified in bronchial biopsies (Appendix-Fig.1A,1B,1C,1D,1E). In some cases (n=22) there was concomitancy of the lesions.
Hyperplasia was defined when more than 4 basal cell layers were observed. Squamous metaplasia was considered when there was replacement of ciliated columnar epithelium by squamous epithelium without atypia. Dysplasia was graded as mild, moderate and severe/CIS according to the level of atypia.

Biopsy products were routinely fixed in 4% neutral buffered formalin immediately after bronchial fibroscopy. They were processed and embedded in paraffin. One paraffin block was chosen from each case, containing several representative fragments. From each paraffin block, several cuts 5µm thick sections were made and put on glass slides then stained with haematoxylin-eosin and reviewed by two pathologists in order to ensure the criteria of the preneoplastic bronchial lesions, according to the 2004 histological WHO/IASLC classification of preinvasive squamous lesions of the bronchi.

METHODS

Immunohistochemistry

The immunohistochemical analysis included seven different antibodies: CK7 (Monoclonal Mouse Anti-human, OV-TL 12/30, Dako); LP34 (Monoclonal Mouse Anti-human, Clone LP34, Dako); Chromogranin A (Monoclonal Mouse Anti-human, Clone DAK-A3, Dako); Ki67 (Monoclonal Mouse Anti-human, Clone MIB-1, Dako); p53 (Monoclonal Mouse Anti-human, Clone DO-7, Dako); C-erbB-2 (Policlonal Mouse Anti-human, Dako) and EGFR (Monoclonal Mouse Anti-human, Clone 31G7, Zymed Laboratories Inc.). The procedure was performed according to a standard avidin-biotin-peroxidase complex.

For each biopsy, external and the intrinsic positive control were considered: basal cells for LP34 and columnar ciliated cells for CK7 in normal bronchial epithelium; carcinoid tumour sections for Chromogranin A; sections of a colonic tubulo-villous adenoma for p53; female breast ductal carcinoma in situ (DCIS) for C-erbB-2 and sections of lung
adenocarcinoma with intense EGFR IHC positivity. Ki67 was validated by a small cell lung carcinoma (SCLC).

LP34, CK7 and Chromogranin A expression were classified as negative or positive. The cut off to classify a lesion as positive for proliferative marker Ki67 was 10% in at least two layers of positive nuclei above the epithelial basal membrane (percentage and intensity of nuclear expression were considered). P53 expression was considered negative or low when there was weak or no nuclear staining, intermediate positivity when there was moderate nuclear expression in less than 50% of the cells and intense positivity when there was an intense nuclear staining or moderate nuclear staining in 50% or more cells in the lesion.

The expression of C-erbB-2 was validated by membrane staining and an identical score to the one used in breast cancer was applied with some alterations because of the limited number of cells observed. Each specimen was scored semi-quantitatively according to the intensity of membrane immunostaining in a 4-point scale: 0/negative - absence of staining; + - weak staining and/or no homogeneous staining; ++ - moderate homogeneous membrane staining; +++ - intense homogeneous membrane staining. For the ++ and +++, we registered the number/percentage of stained cells. Cytoplasmic staining was not considered when present.

A score established by other authors was applied to EGFR protein expression: 0–negative; 1- focal positivity; 2- mild positivity; 3-moderate positivity; 4- intense positivity. The intensity was multiplied by the percentage of positive cells, thus defining a 0-200% score as negative or low expression, 201-300% score as intermediate expression and a 301-400% score as high expression (14).
Fluorescence in situ hybridization (FISH)

Sections of formalin-fixed paraffin embedded specimens, 4 μm thick, were incubated at 65º for 24h, deparaffinized in 2 xylene washes for 10 min and dehydrated in ethanol. The slides were then incubated at 120º for 4 min with 10 mM citrate buffer (pH 6.0), followed by saline sodium citrate buffer (2XSCC pH 7.0) washes. Sections were digested with proteinase K (0.25 mg/ml in 2XSCC, Sigma, pH 7.0) for 5 min at 37º, followed by new 2XSCC wash and post-fixation with 1% formalin for 10 min at room temperature. The slides were later on dehydrated in ethanol series (70-90-100%) for 2 min. Dual-color FISH assays for HER2 were performed by using Qbiogene HER2/neu (17q21)/Alphasatellite 17 dual color probe and for EGFR it was used the Vysis, Inc. USA LSI EGFR Spectrum Orange/CEP 7 Spectrum Green probe. 10 μl probe set was applied to the selected area of the slides and the hybridization areas were covered with a glass cover slip and sealed with rubber cement. The slides were incubated at 90º C, for 6 min, for co-denaturation and at 37ºC for 12-18 h, for hybridization in a humidified chamber. Post-hybridization washes were performed with 50% formamide/2XSCC (pH 7.0) at 46ºC for 5 min, followed by 2XSSC at 46ºC for 2 min. After dehydration in ethanol series, 4’, 6’-diamidino-2-phenylindole (DAPI; 0.15 mg/ml in Vectashield mounting medium, Vector Laboratories, Bulingame, CA, USA) was applied for chromatin counterstaining.

Microscopic analysis was performed on a Nikon Eclipse 80i bright field and epifluorescent microscope equipped with LUCIA cytogenetics software. Fluorescence signals were scored using single-band filters for DAPI, FITC, Texas Red and triple-band pass filter (DAPI, FITC and Texas Red). Images were recorded with a Nikon digital DMX 1200F camera in monochromatic layers which were subsequently merged by Nikon ACT-1 capture software.
The reference slide (stained with haematoxylin-eosin) usually contained normal bronchial epithelium adjacent to the adaptative and preneoplastic lesions and at least 100 non-overlapping interphase nuclei were scored in each fragment for both HER2 and chromosome17 centromer signals and for EGFR and chromosome7 centromer signals, following scoring guidelines and constant adjustments of microscope focus when signals were located at different levels.

Two independent observers performed analysis in a blinded study and scored results, restricted to the selected lesions and bronchial epithelium in each case, with reproducible results.

Four major FISH patterns were identified: balanced dissomy (dissomy for both HER2/chr17 centromer and EGFR/chr7 centromer; balanced trissomy (trissomy for both HER2/chr17 centromer and EGFR/chr7 centromer), balanced polyssomy (≥ 4 gene copies per nucleus in ≥ 40% of cells) and gene amplification (gene copies number/chr number>2 per cell), according to a FISH scoring System defined by Cappuzzo et al (11). Amplification and high polyssomy were considered as FISH positive results.

**Statistical analysis**

Comparisons were performed using bilateral Chi-squared tests or Fisher exact test when needed, because of the small number of lesions in some categories. Anova test was applied to compare the markers expression’s percentage.
RESULTS

**Differentiation markers**

Basal cell hyperplasia had positive LP34 cells in the lower cell layers, while squamous metaplasia showed staining up to the top cell layer, all losing the expression for CK7 (Annex-Table I, Fig.1F and 1G). Dysplasias showed always LP34 positivity and CK7 negativity.

Chromogranin A expression was negative in all the cases (Appendix-Table I).

Caliciform cell hyperplasia, when present, was negative for LP34 (only the basal cell layer remained positive) and maintained the expression for CK7.

**Proliferation (Ki67) and Apoptotic (p53) markers**

Significant differences of Ki67 expression were found between basal cell hyperplasia and squamous metaplasia group and dysplasia group cases (p=0,001). Ki67 expression was higher in dysplasia (p= 0,0007). There was also found a stepwise increment of expression from basal cell hyperplasia, to squamous metaplasia and dysplasia (p<0,0001). The intense expression of Ki67 (intense positive cases) was shown to have a statistical significant increase from basal cell hyperplasia, to squamous metaplasia and dysplasia (p= 0,0002). (Appendix-Table II and III, Fig.1H). Intense positive cases were observed in 19 (57,5%) dysplasias, 9 (22,5%) metaplasias and one basal cell hyperplasia (6,25%). There were no significant differences between the three grades of dysplasia. Despite the intense positive cases observed, moderate expression of Ki67 was the second predominant type of expression in dysplasia (11 cases, 33,3%). Weak expression of Ki67 was mainly observed for basal cell hyperplasia (8 cases, 50%) and squamous metaplasia (13 cases, 32,5%).
An increasing expression for p53 in all the three types of lesions was observed (p<0.0001). P53 expression was significantly higher in dysplasia when compared with basal cell hyperplasias and metaplasias (p=0.0007). Positive intense cases were also more frequent in dysplasia (p=0.0001). In 10 basal cell hyperplasias (62.5%) and 22 squamous metaplasias (55%) it was possible to observe a weak p53 expression. An intense expression of p53 was observed in 20 cases (60.6%) of dysplasia (Appendix-Table II and III, Fig. 1).

EGFR and C-erbB-2/HER2 Protein and Gene expression

EGFR immunohistochemical expression was significantly higher in dysplasias when compared with other preneoplastic lesions (p= 0.009) (Appendix-Table II and III, Fig.1J and 1K, Graphic III). EGFR IHC expression increased between the three groups of lesions (p=0.0005). The intensity of the expression was higher in the group of dysplasia followed by squamous metaplasia, with 18 (54.5%) dysplasias showing moderate expression and 3 cases (9.1%) intense expression, while 14 (35%) squamous metaplasia presented moderate expression and 1 (2.5%) intense expression (p=0.005). Basal cell hyperplasia showed mainly a low EGFR expression, counting 10 (62.5%) cases. Statistical differences between the different grades of dysplasia were not found (Appendix- Graphic III).

C-erbB-2 protein overexpression was clearly observed in only one severe dysplasia (Annex-Table II and III, Fig.1L), without statistical differences between the three groups of lesions or according to the severity of the dysplasia (p=0.14).

C-erbB-2 protein expression was less frequent than C-erB-1.

EGFR and HER2 gene increased copy number was more often due to polissomy than to amplification. Among the 4 cases with intense EGFR protein expression, 3 had high polissomy and 1 showed dissomy.
The number of FISH EGFR positive cases were higher in the group of dysplasia (p=0.0002) (Appendix-Graphic I). Statistical significant correlation between EGFR IHC expression and FISH EGFR results was determined when the intensity of IHQ expression (positive intense vs non intense positive cases) was considered (p=0.0092) (Appendix-Graphic II).

No statistical significant correlation was obtained for IHC c-erbB-2 expression along the spectrum of lesions studied (p=0.14).

HER2 FISH positive cases (n=5) were due to high polissomy (n=4) and high trissomy (n=1). All high polissomy cases were identified in CIS cases and the high trissomy case was identified in a basal cell hyperplasia. These cases were IHC negative. The positive c-erbB-2 case showed low trissomy and considered FISH HER2 negative (Appendix-Fig2A, 2B, 2C).

**DISCUSSION**

As lung carcinogenesis is a multistep process, preneoplastic or preinvasive bronchial lesions’ evolution to lung cancer depends upon the molecular and genetic abnormalities accumulated in time. Those abnormalities increase from the normal bronchial epithelium through basal cell hyperplasia, squamous metaplasia and low grade to high grade dysplasia. Certain proteins and genes play key roles in lung carcinogenesis and their aberrant expression in that process makes them as potential biomarkers susceptible to discriminate a subpopulation of patients with particularly active mutagenesis, progressive cancerization process, at very high risk to develop lung cancer (29).

LP34, CK7, Chromogranin A, Ki67, p53, EGFR and C-erbB2 were the seven biomarkers chosen for this study, as their abnormal expression is related with the oncogenic
process. The first three were used successfully as differentiation markers of the preneoplastic lesions.

A basal cell origin for basal hyperplasia, squamous metaplasia and dysplasia was clearly defined, based on LP34 and CK7 expression. Immunohistochemical differentiation markers like LP34, CK7 and Chromogranin A are useful to discriminate preneoplastic lesions from other proliferative lesions like caliciform cell hyperplasia or neuroendocrine cell hyperplasia. As expected basal cell hyperplasia and squamous metaplasia are always LP34 positive and CK7 and Chromogranin A negative, indicating basal cell origin and conditioning squamous cell carcinoma a basal cell carcinoma. In contrast, caliciform cell hyperplasias maintain CK7 positive cells and are LP34 negative, reflecting an origin in cylindrical cells of the upper cell layer of the respiratory epithelium. Chromogranin A, a neuroendocrine marker, is constantly negative in this study, emphasizing the difference between basal cell hyperplasia and neuroendocrine cell hyperplasia.

Ki67 expression stepwise increment reflects the increasing proliferative index observed between the preneoplastic lesions. The statistical higher expression observed in dysplasia group compared to basal cell hyperplasia and squamous metaplasia defines two groups of lesions according to their proliferative index. As other authors observed, the intensity of Ki67 expression is also higher in dysplasia. This way, we reinforce the utility of Ki67 as a biomarker, namely for dysplasia, as it appears as a group characterized by high proliferative index, subscribing the literature and other authors study of preneoplastic lesions (22,38,6,56,9,42).

P53 is believed to play a role as a “guardian” maintaining the integrity of the genome by participating in the DNA damage checkpoints in the cell cycle, regulating cellular proliferation and apoptosis (34). P53 abnormal expression has been detected in preneoplastic lesions of the lung, suggesting that it occurs early during lung carcinogenesis (18,34,50), as
these results confirm. P53 expression is significantly higher in dysplasia when compared with hyperplasia and metaplasia and the intensity of expression was also higher in the dysplasia group, supporting p53 accumulation involvement in the development of squamous cell lung cancer and fixing p53 as a remarkable biomarker in lung carcinogenesis.

There are many studies concerning EGFR, C-erbB-2, Ki67 and p53 expression in lung cancer. However, there are fewer results validating these markers in preneoplastic lesions and most of them used only immunochemistry techniques. Our study analysed the expression of those biomarkers using not only immunohistochemistry but also applying molecular techniques such as FISH, correlating the results obtained by the two different techniques. By adding FISH to immunohistochemistry, we raised the study to a genetic level, turning those results more reliable.

We demonstrated EGFR immunohistochemical increased expression in preneoplastic lesions, increasing from basal cell hyperplasia to dysplasia, as other studies published have also demonstrated (54,35,16,39,41,49,21,44,57,43). Meert et al (44) also demonstrated not only an increasing EGFR rate from normal bronchial epithelium to CIS and microinvasive tumours, but also a statistically significant cut-off between mild dysplasia and severe dysplasia. Although the greater expression has been seen in dysplasia, there were not statistical differences between the different grades of dysplasia, probably because the number of cases of dysplasia was limited.

EGFR high gene copy number was due more frequently to polyssomy than to amplification. FISH positive EGFR cases are present in all types of preneoplastic lesions and were higher in dysplasia group. Polyssomy appears as an early event in the sequence of hyperplasia-metaplasia-dysplasia. The higher frequency of polyssomy in preneoplastic lesions is reflected in the high frequency of this event in squamous cell carcinomas, constituting the earliest molecular event in lung carcinogenesis (22,16,26).
EGFR protein was overexpressed in all lesions with an increased gene copy number. There was statistical significant correlation between EGFR immunohistochemical expression and FISH EGFR results, when considering the intensity of IHC expression (26). Therefore, it was demonstrated that protein expression does reflect high gene copy number, like polyssomy and amplification. Although EGFR gene amplification is implicated as one mechanism for EGFR overexpression, it doesn’t seem to be the main one (2).

Despite an increasing expression of EGFR and Ki67 along the spectrum of preneoplastic lesions, no correlation between the two markers was found. Meert et al. recently described that low grade lesions showed no EGFR or Ki67 expression contrasting with high grade lesions. EGFR overexpression is associated with cell proliferation, making EGFR an early marker of malignant transformation in lung carcinogenesis (44,49,42).

C-erbB-2 immunohistochemical overexpression is less frequent than C-erbB-1 overexpression, supporting the minor role of C-erbB-2 in squamous cell carcinogenesis when compared with C-erbB-1 (26,45). In our study, only one case of severe dysplasia showed C-erbB-2 positive immunohistochemical expression and polyssomy was observed in 70% of the cells. C-erbB-2 FISH positive cases were due to high polyssomy, showing that amplification is less frequent as a high gene copy number event. No statistical significant correlation was obtained between C-erbB-2 gene and protein expression.

C-erbB-2 does not seem to be involved in the first steps of lung carcinogenesis (47). For Piyathilake et al the expression of C-erbB-2 was significantly higher is squamous cell carcinoma and associated precancerous lesions than in normal epithelium and hyperplastic lesions, but no stepwise expression of C-erbB-2 was observed, suggesting its lack of importance in the squamous cell lung carcinogenesis (49). HER2/neu increased copy number is observed in approximately 35% of adenocarcinomas and slightly less frequently in squamous cell carcinomas (18), assuming more importance in the development of lung
adenocarcinomas. The increased copy number of HER2/neu in NSCLC is most often attributable to chromosome duplication and polyssomy (10), rather than amplification (18).

Although C-erbB-1 and Ki67 expression increases from normal epithelium to preinvasive lesions together, this was not seen for C-erbB-2. Therefore, any correlation between C-erbB-1 and Ki67 expressions and C-erbB-2 expression was established, like other studies also registered (19,52,16,30,44,40). Interestingly, other studies revealed that EGFR/HER2/neu heterodimerization expression is capable to induce a stronger and more sustained proliferative signal than EGFR homodimers (45,57).

The need to accurate detection of the EGFR and HER2/neu gene copy number and protein overexpression, reflecting their importance as biomarkers in squamous cell pathogenesis, has become even more important because despite recent advances in lung cancer treatments, improvement in survival has only been modest, showing that effective therapeutic and early detection approaches are still lacking (49). Selection of patients with preneoplastic lesions expressing selected biomarkers is the way to provide new and more effective strategies for chemoprevention, early diagnosis and targeted treatment with better safety profiles. A variety of new approaches that target selected biomarkers in lung carcinogenesis are in clinical development or have already been approved for second and third line lung cancer treatment (25,32,33,65,27,43,55). Among those approaches are therapeutic agents such as small molecule tyrosine kinase inhibitors (TKis) and monoclonal antibodies (mAbs) specifically targeted against C-ErbB-1 and C-ErbB-2.

Our comparison between gene copy number and protein expression contributes to define which are the most reliable methods to be used in patient selection. Since our study includes a large number of preneoplastic lesions in a different population analysed by two different techniques (IHC and FISH), the results obtained reinforce other results published before, some of them centered only in one molecular marker and/or have used a single
technique, which measures either the protein level or the gene expression. We also stress that our work identifies dysplasia as a group of preneoplastic lesions that could benefit more with chemoprevention targeted schemes. The selection of individual patients for these schemes must consider protein expression, gene copy number and gene mutational status with repercussions in drug sensitivity.

Understanding the expression of specific biomarkers and their pathologic and biologic correlations in lung carcinogenesis, such as the ones here studied, is pivotal to the improvement of diagnosis, treatment and prognosis of lung cancer.

**CONCLUSIONS**

We conclude that squamous cell carcinoma preneoplastic lesions studied have a basal cell origin, as demonstrated by the unbalance between LP34 and CK7. The differentiation markers LP34, CK7 and Chromogranin A successfully discriminate preneoplastic lesions origin.

Dysplasias showed a higher expression of EGFR, Ki67 and p53 with a stepwise expression with the gravity of the preneoplastic lesions, reflecting their importance as potential biomarkers of preinvasive lesions of the bronchial epithelium and may be used to identify patients with an higher risk of developing squamous cell lung carcinoma.

C-erbB-2 immunohistochemical overexpression is a rare event in preneoplastic lesions, emphasizing the minor role of C-erbB-2 as a biomarker in squamous cell carcinogenesis.

EGFR and HER2/neu high gene copy number was present in preneoplastic lesions, with a higher EGFR gene copy number in dysplasias. EGFR and HER2/neu high gene copy
number was due to polyssomy more often than to amplification, reinforcing that amplification is not the main mechanism for protein overexpression. Although HER2/neu does not seem to be involved in the early steps of squamous cell lung carcinogenesis, HER2/neu gene copy number is important because this gene is a HER family member and an important heterodimerization partner for EGFR.

Biological/genetic markers associated with several molecular and genetic abnormalities, such as Ki67, p53, EGFR and HER2/neu, need to be validated as potential and useful preneoplastic bronchial lesion biomarkers. Risk assessment should be based not only on smoking consumption and on histopathology of bronchial lesions, but also on the basis of in situ molecular biomarkers (29). Therefore, in future the recognition of those biomarkers will allow a targeted screening and a posterior closer follow-up to select patients for chemoprevention schemes and will have a positive impact on survival.

REFERENCES


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# APPENDIX

Table I: LP34, CK7 and Chromogranin A expression in preneoplastic lesions

<table>
<thead>
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<th>LP34</th>
<th>CK7</th>
<th>CROMOGRANIN A</th>
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<td>Basal cell hyperplasia n=16</td>
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<td></td>
<td>100%</td>
<td>0%</td>
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<tr>
<td>Squamous Metaplasia n=40</td>
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<tr>
<td></td>
<td>100%</td>
<td>0%</td>
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<td>Dysplasia n=33</td>
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<td>0</td>
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<tr>
<td></td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
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Table II: Ki67, p53, C-erB-b2 and C-erB-b1 intensity of expression in pre-neoplastic lesions

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<tr>
<th></th>
<th>Ki 67</th>
<th>p53</th>
<th>c-ErbB-2</th>
<th>C-Erb B-1</th>
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<tr>
<td></td>
<td>Nº / %</td>
<td>Nº / %</td>
<td>Nº / %</td>
<td>Nº / %</td>
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<tr>
<td>Basal cell hyperplasia (n= 16)</td>
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<td>6.25</td>
<td>1 6.25</td>
<td>15 93.75</td>
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<tr>
<td></td>
<td>+ 9</td>
<td>56.25</td>
<td>10 62.5</td>
<td>1 6.25</td>
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<td>31.25</td>
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<td>0 0</td>
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<td></td>
<td>+++ 1</td>
<td>6.25</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Squamous metaplasia (n= 40)</td>
<td>Neg 8</td>
<td>20</td>
<td>5 12.5</td>
<td>39 97.5</td>
</tr>
<tr>
<td></td>
<td>+ 18</td>
<td>45</td>
<td>22 55</td>
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<tr>
<td></td>
<td>++ 9</td>
<td>22.5</td>
<td>12 30</td>
<td>1 2.5</td>
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<tr>
<td></td>
<td>+++ 5</td>
<td>12.5</td>
<td>1 2.5</td>
<td>0 0</td>
</tr>
<tr>
<td>Dysplasia (n= 33)</td>
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<td>0</td>
<td>1 3</td>
<td>27 81.8</td>
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<tr>
<td></td>
<td>+ 3</td>
<td>9.1</td>
<td>8 24.2</td>
<td>5 15.2</td>
</tr>
<tr>
<td></td>
<td>++ 17</td>
<td>55.5</td>
<td>9 27.3</td>
<td>1 3</td>
</tr>
<tr>
<td></td>
<td>+++ 13</td>
<td>39.4</td>
<td>15 45.5</td>
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</tbody>
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Table III: Ki67, p53, c-erbB-2 and c-erbB-1 expression in preneoplastic lesions considering intensity and percentage of cells with expression

<table>
<thead>
<tr>
<th></th>
<th>Ki 67</th>
<th>p53</th>
<th>c-ErbB-2</th>
<th>C-Erb B-1</th>
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<td>Nº / %</td>
<td>Nº / %</td>
<td>Nº / %</td>
<td>Nº / %</td>
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<tr>
<td>Neg</td>
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<td>1</td>
<td>15</td>
<td>5</td>
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<tr>
<td>Low pos</td>
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<td>10</td>
<td>1</td>
<td>10</td>
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<tr>
<td>Mod pos</td>
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<td>1</td>
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<tr>
<td>Pos int</td>
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<tr>
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<td>19</td>
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<tr>
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<td>27</td>
<td>4</td>
</tr>
<tr>
<td>Low pos</td>
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<td>8</td>
<td>5</td>
<td>8</td>
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<tr>
<td>Mod pos</td>
<td>11</td>
<td>4</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
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<td>Dysplasia</td>
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<tr>
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<td></td>
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<td></td>
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<tr>
<td>Neg</td>
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<td>1</td>
<td>27</td>
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<td>8</td>
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<tr>
<td>Mod pos</td>
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<td>4</td>
<td>1</td>
<td>18</td>
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<tr>
<td>Pos int</td>
<td>19</td>
<td>20</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
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Figure 1: Morphology and immunostaining: Normal bronchial epithelium, H&E, 400x (A); Basal cell hyperplasia, H&E, 400x (B); Squamous metaplasia, H&E, 400x (C); Moderate dysplasia, H&E, 400x (D); In situ carcinoma, H&E, 400x (E); CK7 expression, normal bronchial epithelium, 400x (F); Basal cell hyperplasia, LP34, 400x (G); Ki67 3+ / 70%, 400x (H); P53 2+ / 50%, 400x (I); C-erbB-1, 3+ / 60%, 400x (J); C-erbB-1 negative, normal epithelium, 400x (K); C-erbB-2, 2+, 1000x (L).
Graphic I: EGFR FISH results according to preneoplastic lesions

Figure 2: Dual-color FISH assay with EGFR (red) and chromosome 7 (green). Balanced polysomy, 1000x (A); Dual-color FISH assay with EGFR (red) and chromosome 7 (green). Amplification, 1000x (B); Dual-color FISH assay with HER2neu (red) and chromosome 17 (green), Amplification, 1000x (C).
Graphic II: Correlation between immunohistochemical EGFR expression and FISH results

![Graph showing correlation between EGFR expression and FISH results]

Graphic III: Immunohistochemical EGFR expression between the preneoplastic lesions

![Graph showing EGFR expression in different preneoplastic lesions]