RESEARCH ARTICLE

Impact of HER2 and PTEN Simultaneous Deregulation in Non-small Cell Lung Carcinoma: Correlation with Biological Behavior

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Abstract

Background: HER2/neu overexpression due to gene amplification is an important factor in breast cancer, modifying the sensitivity to anti-HER2 monoclonal antibody therapy. The clinical significance of HER2 expression in non small cell lung carcinoma (NSCLC) is currently under evaluation. The tumor suppressor gene PTEN negatively regulates the HER2/PI3K/Akt signalling pathway. The purpose of this study was to evaluate the role of simultaneous alteration in HER2 and PTEN protein expression in relation to biological behaviour of NSCLCs. Materials and Methods: Protein expression was determined by immunohistochemistry in sixty-one (n=61) NSCLC cases along with CISH for HER2 gene analysis and detection of chromosome 17 aneuploidy. Patients were followed-up for a period of 34 to 41 months after surgery. Results: HER2 overexpression (2+/3+ score) was detected in 17 (27.9%) patients while loss of PTEN expression was observed in 24 (39.3%) cases, low expression in 29 (47.6%) and overexpression in 8 (13.1%). Simultaneous HER2 overexpression and PTEN low/loss of expression were correlated with metastasis (71.4% vs 36.2% p=0.03). Analysis in the subgroup of 22 patients of pTNM stage III with lymph node status N1 or N2 revealed that there was a relationship between the number of positive regional lymph node groups and simultaneous deregulation of the two genes (p=0.04). Multivariate analysis determined that HER2 overexpression was associated with an increasing risk of developing metastases (OR: 4.3; 95%CI: 1.2-15.9; p: 0.03) while PTEN overexpression was associated with lower risk (OR: 0.1; 95%CI: 0.1, 1.0; p: 0.05). Conclusions: Simultaneous HER2/PTEN deregulation is a significant genetic event that leads to a more aggressive phenotype of NSCLC.

Keywords: HER2/neu - PTEN - non-small cell lung carcinoma - gene

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Introduction

Lung cancer is the leading cause of cancer death worldwide in both men and women, with an estimated 1.4 million deaths each year (Jemal et al., 2011). Non-small-cell lung cancer (NSCLC) accounts for 80-85% of all lung cancer cases (Sher et al., 2008). When feasible, surgical resection remains the single most consistent and successful option for cure. However, close to 70% of patients with lung cancer present with locally advanced or metastatic disease at the time of diagnosis (Molina et al., 2008). The prognosis for patients with NSCLC is strongly correlated with disease stage at the time of diagnosis with 5-year survival rate of about 60% for clinical stage I to less than 5% for clinical stage IV. Improving the survival rate of patients with this disease requires a better understanding of tumor biology and the subsequent development of novel therapeutic strategies. In recent years, a number of molecular markers – including HER2/neu and PTEN – have been tested as predictors for survival and response to cytotoxic chemotherapy and multimodality treatment.

The HER2/neu protooncogene is located on the long arm of chromosome 17 (17q21), encoding for a transmembraneous glycoprotein HER2 with intrinsic tyrosine kinase activity and marked sequence homology with the epidermal growth factor receptor (EGFR). Dimerization of HER 2 with an activated EGFR molecule leads to the activation of a signal transduction cascade with subsequent increase in angiogenesis, cell proliferation and metastatic potential, as well as a decrease in apoptosis (Citri and Yarden, 2006). HER2/neu is expressed in a wide variety of human epithelial malignancies including breast,

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ovary, salivary glands, gastrointestinal tract, prostate, kidney, liver and lung suggesting that its overexpression could play a critical role in the development and progression in those cancers (Meert et al., 2003). In NSCLC, the HER2/neu gene activation prevalence, its prognostic role and possible therapeutic implications are still under study (Cagle and Chirieac, 2012).

The tumor suppressor PTEN is located on chromosome 10q23 encoding for a lipid phosphatase that antagonizes signal transduction downstream of PI-3 (phosphatidylinositol-3) kinase by dephosphorylating phosphatidylinositol-triphosphate (PtdInsP) and suppresses cell growth through the negative regulation of cell cycle and cell survival. Downregulation of PTEN is associated with increased PI-3 kinase activity with subsequently higher levels of 3′-phosphorylated phosphoinositides, which bind to and activate Akt. Activated Akt promotes cell survival by phosphorylating and modulating the activity of various transcription factors (Shoman et al., 2005; Song et al., 2012). Germline mutations of PTEN are associated with autosomal dominant hamartomatous and often, cancer-prone syndromes while homozygous inactivation of PTEN has been found in a wide spectrum of sporadic human cancers (Chu and Tarnawski, 2004). Reduction and loss of PTEN protein expression has been noted in primary tumors with frequencies ranging from 20% in gastric carcinomas to almost 70% in NSCLC (Kang et al., 2002; Marsit et al., 2005). However, the role of PTEN in patients with NSCLC has not been well established and further studies are needed to evaluate its prognostic role.

On the basis of the data listed above, we have postulated here that the simultaneous deregulation of PTEN and HER2 genes contribute to carcinogenesis. We examined immunohistochemically the expression pattern of HER2 and PTEN proteins in NSCLCs and we determined if these alterations have any effect on disease progression.

Materials and Methods

Patients/Tissue samples

Sixty-one (n=61) consecutive patients who had been diagnosed with stage I-IIIA NSCLC underwent complete pulmonary resection (lobectoby or pneumonectomy) and systematic lymph node sampling (routine excision biopsy from all accessible stations depending on the side). We used for the purposes of our study their formalin fixed and paraffin embedded tissue samples of histologically confirmed NSCLCs. The Department of Pathology (Sotiria Chest Diseases Hospital of Athens, Greece) the local ethical committee gave permission to use those tissues for research purposes. Written informed consent was obtained from each patient and the study protocol conforms to the ethical guidelines of the "World Medical Association Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects" adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, as revised in Tokyo 2004. All corresponding Hematoxylin and Eosin (H&E)-stained slides were reviewed by two pathologists for the confirmation of diagnosis and classification according to World Health Organization (WHO, 2000) grading criteria and TNM7 system. The tissue samples were referred to 49 male and 12 female (mean age: 63.1±8.96) patients followed-up in a period of 34 to 41 months after the operation. The postoperative follow-up consisted of screening chest, brain and upper abdomen CT and blood work at intervals dependent on the pathological stage (pTNM). Among the 61 participants 27 (44.3%) developed metastasis (intra or extra thoracic). Clinicopathological data are demonstrated in Table 1.

Antibodies and probes

Ready-to-use HER2 monoclonal mouse antibody (clone TAB250-Zymed/InVitrogen, San Fransisco, USA) recognizing predominantly the extracellular domain of HER2 protein and not reacting with other erbB receptors was applied for the identification of protein expression. Additionally, rabbit polyclonal anti-PTEN (PN37- InVitrogen/Zymed, USA) was applied in the corresponding cases. HER2 gene status was determined using the ready to use SPOT LIGHT HER2 DNA Probe (Zymed/InVitrogen, San Fransisco, USA). This digoxygenin-labeled probe is located on 17q21 and covers the entire HER2 gene area. Similarly, chromosome 17 status was determined by the ready to use biotin-labeled chromosome 17 centromeric probe (Zymed/InVitrogen, San Fransisco, USA) recognizing the specific repetitive centromeric DNA sequences known as α-satellite DNA.

Immunohistochemistry (IHC)

IHC for HER2 and also PTEN antigens was carried out on 3 μm serial sections of the corresponding tissue blocks. Two slides were deparaffinized and rehydrated. Both of them were enzyme digested (proteinase K) for 10 min at 37ºC. The NBA kit (Zymed/InVitrogen, San Fransisco, USA) was used for the following detection steps. Blocking solution was applied to the slides for 10 min, followed by incubation for 1 h using the antibodies (dilution 1:20 and 1:60, respectively) at room temperature. Following incubation with the secondary antibody for 10 min, diaminobenzidine-tetrahydrocloride-DAB (0.03%) containing 0.1% hydrogen peroxide was applied as a chromogen and incubated for 5 min. Sections were counterstained, dehydrated and cover-slipped. For negative control slides, the primary antibodies were omitted. IHC protocol was performed by the use of an automated staining system (I 6000 – Biogenex, San Ramon, CA, USA). Membranous predominantly and sub-membranous cytoplasmic staining was considered acceptable for HER2 expression according to the manufacturer's data sheet (Figure 1a-c), whereas diffuse cytoplasmic staining was evaluated regarding PTEN expression (Figure 1d-g). Breast cancer tissue sections overexpressing HER2 and PTEN proteins and normal appearing lung epithelia were used as a positive and negative control, respectively. Protein expression levels were evaluated semi-quantitatively by using Zymed's Evaluation Guidelines. According to the scoring guidelines, the examined cases were classified as follows: Score 0: no staining or membrane staining in <10% of tumor cells; Score 1+: faint membrane staining in >10% of tumor cells; Score 2+: weak or moderate

Table 1. Clinical Data and Combined HER2 and PTEN ICH Analysis

Variables		HER2 Expression			PTEN Expression			
		No/Low (0/1+) (N=44)	High (2+/3+) (N=17)	P value	No (0) (N=24)	Low (1+) (N=29)	High (2+) (N=8)	P value
Age (years)		63.0±9.33	63.2±8.19	0.95	63.3±7.93	64.3±8.20	57.8±13.2	0.18
Gender				0.99				0.07
	Male	35 (79.6)	14 (82.4)		17 (70.8)	24 (82.8)	8 (100.0)	
	Female	9 (20.4)	3 (17.6)		7 (29.2)	5 (17.2)	0 (0.00)	
Tumor size (cm)		4.88±2.79	5.32±2.9	0.58	5.32±2.88	4.57±2.73	5.63±2.94	0.50
pTNM Stage				0.17				0.18
	I	20 (45.6)	4 (23.5)		6 (25.0)	14 (48.3)	4 (50.0)	
	II	9 (20.4)	5 (29.4)		8 (33.3)	4 (13.8)	2 (25.0)	
	III	15 (34.0)	8 (47.1)		10 (41.7)	11 (37.9)	2 (25.0)	
Lymph node status				0.17				0.84
	N0	25 (56.8)	7 (41.2)		12 (50.0)	16 (55.2)	4 (50.0)	
	N1	9 (20.5)	3 (17.6)		5 (20.8)	5 (17.2)	2 (25.0)	
	N2	10 (22.7)	7 (41.2)		7 (29.2)	8 (27.6)	2 (25.0)	
Histol. subtype				0.47				0.49
	Adeno	24 (54.6)	11 (64.7)		14 (58.3)	18 (62.1)	3 (37.5)	
	Squamous	20 (45.4)	6 (35.3)		10 (41.7)	11 (37.9)	5 (62.5)	
Grade				0.41				0.25
	Low	26 (59.1)	12 (70.6)		14 (58.3)	17 (58.6)	7 (87.5)	
	Med./High	18 (40.9)	5 (29.4)		10 (41.7)	12 (41.4)	1 (12.5)	
Chemotherapy				0.74				0.88
	No	11 (25.0)	3 (17.7)		6 (25.0)	6 (20.7)	2 (25.0)	
	Yes	33 (75.0)	14 (82.3)		18 (75.0)	23 (79.3)	6 (75.0)	
Metastasis				0.04				0.14
	No	28 (63.6)	6 (35.3)		12 (50.0)	15 (51.7)	7 (87.5)	
	Yes	16 (36.4)	11 (64.7)		12 (50.0)	14 (48.3)	1 (12.5)	
Time to meta (months)*		15.1±6.34	16.4±7.96	0.51	15.3±7.07	16.1±7.23	12.00±0.0	0.71
Survival				0.42				0.52
	Yes	33 (75.0)	11 (64.7)		17 (70.8)	20 (69.0)	7 (87.5)	
	No	11 (25.0)	6 (35.3)		7 (29.2)	9 (31.0)	1 (12.5)	
Time to death (months)**		19.4±7.14	21.7±7.08	0.39	18.0±7.43	21.3±6.91	25.0±0.00	0.50
Smoking (py)		71.4±42.2	77.4± 42.0	0.62	74.4±37.0	72.3±47.7	71.5±38.0	0.98

Data are presented as N (%) or mean±standard deviation, *Variable refers to the subgroup of 27 patients who developed metastasis, **Variable refers to the subgroup of 17 patients who died

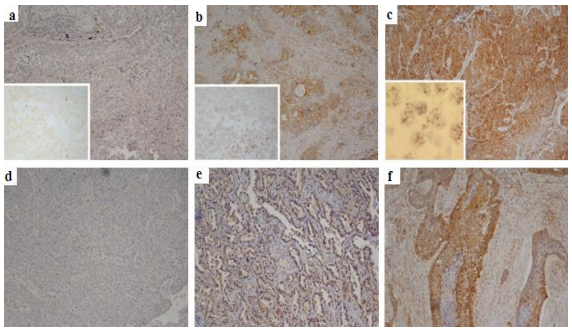

Figure 1. HER2 and PTEN Protein Expression Patterns in NSCLC. HER2 low expression (1+), moderate expression (2+) and overexpression (3+) in NSCLC cases, respectively (1a-c). Note in 3+ score, a dense, continuous membrane staining. HER2 gene status detected by CISH analysis. Normal diploid and low amplification in 1+, a 2+ and 3+ cases, respectively (1a-c, inside images). PTEN low expression (1+), moderate expression (2+) and overexpression (3+) in NSCLC cases, respectively (1d-f). Original magnification 10x (IHC) and 40x (CISH)

complete membrane staining in >10% of tumor cells and Score 3+: strong, complete membrane staining in >10% of tumor cells. Scores of 0 and 1+ were considered as negative for HER2 expression while Scores 2+ and 3+ as positive (overexpression). Additionally, complete absence of stain regarding PTEN was considered as 0 score, moderate expression levels as 1+, whereas strong expression validated as 2+/3+ score.

Chromogenic in situ hybridization (CISH)

CISH SPOT-Light Chromogenic ISH Detection Kit (Zymed/InVitrogen, USA) was applied. CISH for chromosome 17 and HER2 gene analysis was performed on 5 μm thick paraffin serial sections of the tissue blocks described above. Two slides were incubated at 37oC overnight followed by 2 h incubation at 60ºC and then deparaffinized in xylene two times, 5 min each and in ethanol three times, 3 min each. The slides were rinsed in deionised water and then placed in a coplin jar containing CISH FFPE Pre-treatment Buffer (CISH Tissue Pre-treatment Kit, Zymed). For heat pre-treatment, the coplin jar was capped, loosely screwed, placed in a pressure cooker and timed for 10 min after the pressure built up. The slides, then, were immediately washed in deionised water followed by enzyme digestion, which was performed by covering the sections with pepsin (CISH Tissue Pre-treatment Kit, Zymed) for 5 min at 37ºC. The slides were washed with deionised water, dehydrated with graded ethanol and air-dried. Ready to use dig-labeled HER2 gene and biotin-labeled chromosome 17 centromere probes were applied to each section, respectively. Twenty microliter of probe was applied to section. The tissue sections containing the added probe were denatured by placing the slides in a polymerase chain reaction (PCR) machine equipped with a slide block at 94°C for 5 min. The slides were then placed in a moist slide box and incubated at 37°C for overnight hybridization. The

sections were stringently washed in 0.5x standard saline citrate at 75°C for 5 min. The CISH Polymer and the Horseradish (HRP) Detection Kit (Zymed/InVitrogen, San Fransisco, USA) – containing similar steps to IHC – were used. Shortly, afterwards TMA sections were placed in 3% H_2O_2 and diluted with methanol for 10 min to block endogenous peroxidase. To block unspecific staining, Cas BlockTM (Zymed/InVitrogen, San Fransisco, USA) was applied and incubated for 10 min. Following incubation with mouse anti-dig for 30 min and then polymerised HRP conjugated anti-mouse for 30 min, the HER2 probe was visualized by DAB development (CISH Polymer Detection Kit, Zymed). The biotin labeled Chr 17 centromere probe was detected by incubation with HRP conjugated streptavidin for 30 min, followed by DAB development (CISH Centromere Detection Kit, Zymed) for 30 min. Tissue sections were lightly counterstained with hematoxylin and dehydrated in graded ethanol. At the end of the process, CISH centromere signals or gene copies were easily visualized as dark brown/blue scattered or in small clusters dots, using a conventional, bright-field microscope (Figure 1a-c, inside). Interpretation of HER2 gene and chromosome 17 centromere signal results was based on Zymed's Evaluation Chart for CISH. According to this guide, two gene copies per nucleus demonstrate normal HER2 gene pattern, whereas 6-10 or small clusters characterize a low-level gene amplification. In this case, chromosome 17 status must be evaluated to exclude aneuploidy (3-5 centromeric signals per nucleus; diploid pattern demonstrates normal chromosome status). High gene amplification level is characterized by the presence of more than 10 gene copies or large clusters of them per nucleus in more than 50% of the examined cells, whereas the presence of a smaller number of HER2 copies than chromosome 17 centromeric signals is considered to be an evidence of gene deletion or ''silence'' due to mechanisms, such as point mutation or loss of heterozygosity (LOH).

Statistical analysis

Continuous data are presented as mean±standard deviation whereas categorical data as absolute and relative frequency. T-test for continuous data in two independent samples, ANOVA technique for continuous data in more than two independent samples, χ^2-test for categorical data and Fisher's Exact test for categorical data with limited number of frequencies were applied. Kaplan-Meier and Cox proportional hazards ratio were used to explore the relationship of study parameters with survival. Significance level was set at p=0.05. The SAS statistical package (Version 9.1, SAS Institute Inc, Cary, NC) was used to analyze the data.

Results

The study sample consisted of 61 patients diagnosed with primary NSCLC. Among them, 49 (80.3%) were male and 12 were female (19.7%), with a mean age of 63.1±9.0 years, ranging between 39 and 78 years. Table 1 presents socio-demographic characteristics and medical history of study participants according to the expression of genes HER2 and PTEN. From the total sample of 61 patients, 24 (39.3%) were classified as pTNM stage I, 14 (23.0%) as pTNM stage II and 23 (37.7%) as pTNM stage III. 35 (57.4%) patients had adenocarcinoma and 26 (42.6%) had squamous cell carcinoma. We detected HER2 overexpression in 17 (27.9%) patients. With regard to gene amplification, we found that no patient showed a true high gene amplification and only 4 patients (6.55%) had low gene amplification, of which three had adenocarcinoma and one squamous cell carcinoma. Among the patients with adenocarcinoma and low gene amplification 1 had HER2(+3) overexpression (the only one patient in the study with +3 expression), 1 had HER2(+2) overexpression and 1 had weak HER2(+1) expression. The patient with squamous histopathology and low gene amplification had weak HER2(+1) expression too. Regarding PTEN, loss of PTEN expression was observed in 24 (39.3%) cases, low expression in 29 (47.6%) and overexpression in 8 (13.1%) cases.

Univariate analyses

There was no evidence of association between HER2 and PTEN expression (p=0.30). Specifically among the 17 patients who expressed high levels of HER2, we found that 5 (29.4%), 9 (52.9%) and 3 (17.7%) patients showed no, low and high PTEN expression respectively. Among those 44 cases with weak expression of HER2, 19 (43.2%), 20 (45.5%) and 5 (11.3%) showed no, low and high PTEN expression levels, respectively.

HER2 expression was not found to be related with age (p=0.95), gender (p=0.99), tumor size (p=0.58), pTNM stage (p=0.17), lymph node status (p=0.17), histologic subtype (p=0.47), grade (p=0.41), submission to chemotherapy (p=0.74), survival rates (p=0.42) and smoking (p=0.62). On the other hand, there was evidence for a positive relationship of HER2 with occurrence of metastasis (p=0.04). Specifically, metastasis was detected in 64.7% of patients who overexpressed HER2 and in 36.4% of patients with weak HER2 expression. 11 patients with weak HER2 expression died during the study; 8 (72.7%) were of pTNM stage III and 9 (81.8%) were of low grade. 6 patients with HER2 overexpression died during the study; 4 (66.7%) were of pTNM stage III and 5 were (83.3%) of low grade. Among the 16 participants with weak HER2 expression who developed metastasis, 10 (62.5%) were of pTNM stage III and 12 (75.0%) had low grade. In the group of 11 patients with HER2 overexpression who developed metastasis, 8 (72.7%) were of pTNM stage III and 8 (72.7%) had low grade.

Concerning the total sample of 61 participants, 24 (39.3%) were characterized by loss of PTEN expression, 29 (47.6%) had low PTEN expression and 8 (13.1%) had high PTEN expression. There were no significant differences in the expression of PTEN in relation to age (p=0.18), gender (p=0.07), tumor size (p=0.50), pTNM stage (p=0.18), lymph node status (p=0.84), histologic subtype (p=0.49), grade (p=0.25), submission to chemotherapy (p=0.88), survival rates (p=0.52) and smoking (p=0.98) (Table 1). Although patients with loss or low PTEN expression seem to develop metastasis more frequently as compared to patients with PTEN overexpression, the difference was not finally statistically significant (p=0.14). 7 patients with

loss of PTEN expression died during the study; 5 (71.4%) were of pTNM stage III and 5 (71.4%) were of low grade. 9 patients with low expression of PTEN died during the study; 6 (66.7%) were of pTNM stage III, 8 were (88.9%) of low grade. The one patient with overexpression of PTEN who died during the study was of pTNM stage III and low grade. Among the 12 patients with loss of PTEN expression who developed metastasis, 8 (66.7%) were of pTNM stage III and 8 (66.7%) were of low grade. In the group of 14 patients with low expression of PTEN who developed metastasis, 9 (64.3%) were of pTNM stage III, 11 were (78.6%) of low grade. The one patient with PTEN overexpression who developed metastasis was of pTNM stage III and low grade. Furthermore we attempted an alternative approach, by merging two of the three levels of PTEN expression, but the results were similar with the previous ones. Finally, we explored the simultaneous expression of HER2 and PTEN by creating two groups: (a) the first group included patients with no/low PTEN expression (PTEN: 0/1) and overexpression of HER2 (HER2: 2/3), and (b) the second group included all the remaining categories. Simultaneous HER2 overexpression and no/low PTEN expression was reported in 14 participants (22.9%). Apart from the occurrence of metastasis (71.4% of the first group developed metastasis vs. 36.2% in the second group; p=0.03), none of the study variables was found to differ significantly between the two groups (Table 2).

Table 2. Clinical Data and Simultaneous HER2 and PTEN Deregulation

Variables	PTEN (0/1) & HER2(2/3) (n=14)	All remaining categories (n=47)	P-value
Age (years)	63.4±7.50	63.0±9.42	0.86
Gender			0.99
Male	11 (78.6)	38 (80.8)	
Female	3 (21.4)	9 (19.2)	
Tumor size (cm)	5.29±2.91	4.92±2.78	0.67
pTNM Stage			0.07
I	2 (14.3)	22 (46.8)	
II	5 (35.7)	9 (19.2)	
III	7 (50.0)	16 (34.0)	
Lymph node			0.12
N0	5 (35.7)	27 (57.5)	
N1	3 (21.4)	9 (19.1)	
N2	6 (42.9)	11 (23.4)	
Histologic subtype			0.36
Adeno	10 (71.4)	25 (53.2)	
Scuamous	4 (28.6)	22 (46.8)	
Grade			0.86
Low	9 (64.3)	29 (61.7)	
Medium/High	5 (35.7)	18 (38.3)	
Metastasis			0.03
No	4 (28.6)	30 (63.8)	
Yes	10 (71.4)	17 (36.2)	
Time to metastases (months)*	16.8±8.25	14.9±6.18	0.41
Survival			0.46
Yes	9 (64.3)	35 (74.5)	
No	5 (35.7)	12 (25.5)	
Time to death (months)**	21.0±7.71	19.8±7.00	0.61
Smoking (py)	74.5±40.3	72.6±42.7	0.88

Data are presented as N (%) or mean±standard deviation, *Variable refers to the subgroup of 27 patients who developed metastasis, **Variable refers to the subgroup of 17 patients who died

In addition, we performed a further analysis in the subgroup of 29 cases with lymph node status N1 or N2. For these patients we counted the number of positive regional lymph node groups during the time of surgery according to the histology report. (When two or more nodes from the same group were positive that was accounted for as one positive group). Indeed, there was a strong evidence for relationship between the number of positive regional lymph node groups and simultaneous deregulation of the two genes (p=0.001). Among the 9 cases with no/low PTEN expression and HER2 overexpression, 1 (11.1%) had 1 positive group, 2 (22.2%) had 2 positive groups and 6 (66.7%) had 3 positive groups. Among the remaining 20 cases, 13 (65.0%) had 1 positive group, 6 (30.0%) had 2 positive groups and 1 (5.0%) had 3 positive groups. But the 29 patients with lymph node status N1 or N2 belonged to a heterogeneous group according to the pTNM stage. 22 of them were of stage III and 7 were of stage II. (in this study the total number of patients of stage III was 23, but the patients of stage III with N1 or N2 disease were 22 – we excluded from the stage III subgroup a patient with T4N0M0 disease). In order to avoid erroneous conclusions due to differences in the TNM stage we excluded the patients of pTNM stage II because of the limited number and focused only in the subgroup of 22 patients of pTNM stage III with N1 or N2 disease. According to the results of the Fisher's Exact Test there was again evidence for a statistically significant correlation between the number of positive regional lymph node groups and simultaneous deregulation of the two genes (p=0,04). Among the 7 patients with no/low PTEN expression and HER2 overexpression 1(14.3%) had 1 positive lymph node group, 2(28.6%) had 2 positive groups and 4(57.1%) had 3 positive groups. Among the remaining 15 patients 8(53.3%) had 1 positive group, 6(40%) had 2 positive groups and 1(6.7%) had 3 positive groups (Figure 2).

Multivariate analyses

To explore further possible associations of the simultaneous expression of HER2 and PTEN with patients' output, we modeled the data through multiple (linear or logistic) regressions. In all these models HER2 and PTEN are simultaneously introduced as independent

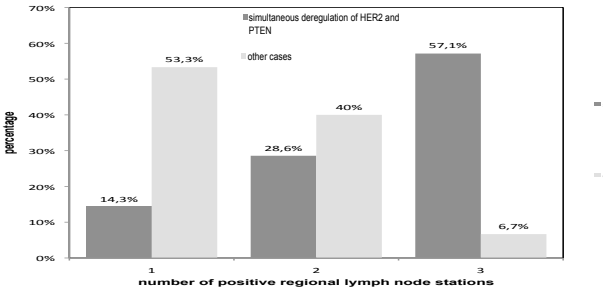

Figure 2. Distribution of 22 Patients of Stage III with N1 and N2 Disease According to the Number of Positive Regional Lymph Node Groups and Gene Expression. There was evidence for a statistically significant correlation between the number of positive regional lymph node groups and simultaneous deregulation of the two genes (p=0.04)

variables whereas a series of study parameters reflecting patients' prognosis are added as dependent variables one at a time. There was no indication that HER2 is associated with tumor size (p=0.57), histologic subtype (p=0.42), grade (p=0.47) and survival (p=0.37). On the other hand we found that HER2 overexpression is associated with increasing risk of developing metastasis as compared to underexpression of HER2 (OR: 4.3; 95%CI: 1.2-15.9; p:0.03). Although HER2 overexpression seems to double approximately the risk of increasing pTNM stage (OR: 2.3; 95%CI: 0.8-6.9), the result did not reach statistical significance (p=0.12). Similarly, HER2 overexpression seems to double the risk of increasing lymph node status (OR: 2.2; 95%CI: 0.8-6.5), but once again the estimation of p (p=0.15) was above the level of statistical significance.

Regarding PTEN, there was no evidence for a possible association of its expression with the clinical outcomes, apart from a borderline evidence for the development of metastasis. Specifically, those with high PTEN expression as compared to those with no/low PTEN expression have lower risk to develop metastasis (OR: 0.1; 95%CI: 0.1, 1.0; p: 0.05). When we repeated the multivariate analyses controlling also for gender and age the results were similar.

Survival analysis

According to the findings of survival analysis, there was no significant evidence for univariate association of patients' survival with none of the following variables: PTEN expression (p=0.50), HER2 expression (p=0.60) and histologic subtype (p=0.25). On the other hand we found that poor survival was significantly associated with the occurrence of metastasis (p=0.002) and increasing levels of tumor size (p=0.01), pTNM stage (p=0.01) and lymph node status (p=0.01).

Discussion

Carcinogenetic process regarding NSCLC is based on a multigenetic abnormity. It has been widely accepted that both activation of protooncogenes, and inactivation of tumor suppressor genes, due to amplification or point mutations/deletions, respectively play a key role in the genesis and progression of NSCLC (Tang et al., 2006). The overexpression of HER-2/neu in NSCLC appears to be a prognostic factor for an unfavourable course of the disease, a reduced overall survival and shorter progressive free periods (Tan et al., 2003; Au et al., 2004; Liu et al., 2010; Takenaka et al., 2011). However, those findings were not verified in some studies and the importance of this prognostic biological factor will probably be small in comparison with other factors such as TNM stage, age or performance status (Meert et al., 2003; Pelosi et al., 2005).

In the current study, we found HER-2/neu overexpression (+2,+3) by IHC in 27,9% of the NSCLC tumors (31,4% in adenocarcinomas and 23,1% in in squamous cell cancers) and these results are similar to most reports in the literature (7-32%) (Swanton et al., 2006; Timotheadou et al., 2007). HER-2/neu gene amplification seems to be a rare event in NSCLC and the cell surface expression of HER-2/neu is most often attributable to increased copy number from chromosome duplication and polysomy (Nakamura et al., 2003). Indeed, in the analyzed patient population we found no case with true amplification but only low gene amplification in 4 patients (6.55%) 3 of which was adenocarcinomas. Furthermore, in two of them, low-level gene amplification was associated with faint HER-2/neu immunoreactivity (+1).

Concerning PTEN, its role in NSCLC has not been convincingly demonstrated, but according to several studies there is sufficient association between PTEN expression and the clinical behaviour of lung cancer. There were many reports where loss of PTEN expression is involved with invasion, metastasis and with survival disadvantage in NSCLC (Lim et al., 2007; O′ Byrne et al., 2011; Yoo et al., 2011; Wang et al., 2012). Although there were other studies with the inconsistence reports that PTEN expression is not associated with prognosis in patients with NSCLC (Olaussen et al., 2003), the established function of PTEN in controlling the phosphorylation status of multiple proteins with crucial roles in cell biology strongly supports a key role for this gene in the pathogenesis of lung cancer. Genetic alterations of the PTEN gene are rare in NSCLC but loss of PTEN protein is not an uncommon event (Bepler et al., 2004; Jin et al., 2010; Lee et al., 2010). In this study we found loss or low PTEN expression in 86.9% of patients while only PTEN overexpression was associated with lower risk to develop metastases (p=0.05). We reported here that there was no evidence of association between HER2 and PTEN expression (p=0.30), however there is a significant number of patients (22.9%) with simultaneous HER2 overexpression (+2,+3) and no/low PTEN expression (0,+1). In this subgroup we found higher occurrence of metastasis (71.4%, p=0.03) but not any association between survival and PTEN (p=0.50), survival and HER2 (p=0.60) or survival and simultaneous HER2 and PTEN deregulation (p=0,46) and this could be explained by the small follow up time of this study (34-41 months). Furthermore, among the patients of pTNM stage III with lymph node status N1 or N2 during the time of surgery the patients with simultaneous HER2 overexpression and no/low PTEN expression had a larger number of positive regional lymph node groups (p=0.04) than those without simultaneous deregulation. It seems that these patients had a potentially larger local distribution of the disease.

Trastuzumab has been a model of a rationally designed, highly specific, targeted cancer therapy and has brought valuable therapeutic benefits to patients with HER-2/neu overexpressing cancers (Valabrega et al., 2007). However, even in patients with tumors expressing very high levels of HER2, there are limited response rates of trastuzumab and PTEN loss could be responsible for this (Wang et al., 2011). According to Nagata trastuzumab specifically downregulates PI3K signaling through PTEN activation (Nagata et al., 2004). When present in the plasma membrane, PTEN dephosphorylates the lipid products of PI3K and prevents activation of key PI3K targets. PTEN binds to the plasma membrane via the C2 domain that is negatively regulated by Src-dependent tyrosine phosphorylation. Trastuzumab binding rapidly decouples Src from HER2 receptor. The consequent loss

of Src activity reduces PTEN C2 domain phosphorylation and allows PTEN to translocate to the plasma membrane and inhibit the PI3K pathway. The mechanism of trastuzumab function via inactivation of Src, clearly indicates that trastuzumab responsiveness depends not only on the downregulation of HER2 and the inhibition of HER2 related downstream events, but also on the status of PTEN. In breast cancer patients, those with HER2 gene amplification and IHC +3 have the higher response rate in treatment inclunding trastuzumab (Perez et al., 2010). In NSCLC most data about deregulation of HER2 have emerged mainly from the study of tumors with immunohistochemistry (Hirsch et al., 2002; Vogel et al., 2002) while the patients whose tumors had HER2 gene amplification and who were treated with trastuzumab had a response to trastuzumab (Gatzemeier et al., 2004). The results of the present study suggest that the patients that not expected to have good response to trastuzumab (HER2 +2/+3, PTEN 0/+1), are these who have the worse biological behavior (higher regional lymph node load at the time of operation and higher occurrence of metastases). For these patients seems necessary a different therapeutic approach. Based on the fact that the efficacy of trastuzumab is dependent on the ability to inhibit PI3K signaling through activation of PTEN it seems logical that the efficacy of trastuzumab could be enhanced with inhibitors of the PI3K pathway. On the basis of the preclinical data generated in mouse and rat models, a therapeutic window for PI3K inhibitors exists (Maira et al., 2009). Phase I study of BKM120, an oral pan-Class I PI3K inhibitor, has indicated the feasibility and proof-of-concept of class I PI3K inhibition in patients with advanced tumors (Bendell et al., 2012).

In conclusion, simultaneous HER2 and PTEN deregulation is a significant genetic event that is likely to offer in the progression of NSCLC. In patients with this specific genetic profile a rational targeted multi-level therapeutic approach must be evaluated.

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