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ORIGINAL ARTICLE

PTEN expression in non small cell lung carcinoma based on digitized image analysis

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Summary

Purpose: *HER2 depended signalling pathway is deregulated in a subset of non small cell lung carcinoma (NSCLC). The tumor suppressor gene PTEN (10q21) regulates the HER2/PI3K/Akt signalling pathway. Our aim was to evaluate PTEN protein expression in NSCLC based on a quantitative analysis method correlating also the results with clinicopathological parameters.*

Methods: *Protein expression was determined by immunohistochemistry (IHC) in 61 paraffin-embedded cases of patients with NSCLC. Digital image analysis (staining intensity levels) was performed in the corresponding immunostained slides.*

Results: *Loss of PTEN expression was observed in 24 (39.34%) cases, low expression in 29 (47.54%) and overexpression in 8 (13.12%) cases. Multivariate analysis determined that PTEN overexpression was associated with lower risk to develop metastases (p=0.05).*

Conclusion: *PTEN deregulation is a relatively frequent genetic event in NSCLC, associated with progressive metastatic process in those patients. Because of binding to the ErbB2 receptor, trastuzumab stabilizes and activates PTEN gene, and loss of its expression negatively affects the response rates in such patients.*

Key words: image analysis, immunohistochemistry, lung carcinoma, PTEN, suppressor genes

Introduction

The tumor suppressor gene 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 negative regulation of cell cycle and cell survival [1]. Downregulation of PTEN is associated with increased PI-3 kinase activity with subsequent higher levels of 3´-phosphorylated phosphoinositides, which bind to and activate Akt [2,3]. Activated Akt promotes cell survival by phosphorylating and modulating the activity of various transcription factors [4,5]. 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 [6]. 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 [7,8]. However, the role of PTEN in patients with NSCLC has not been well established and further studies are needed to evaluate its prognostic role.

In the current study we evaluated PTEN protein expression in NSCLC specimens, based on digital image analysis correlating it with clinicopathological parameters.

Methods

Patients/Tissue samples

Sixty-one formalin-fixed and paraffin-embedded archival tissue samples of histologically confirmed NSCLCs were used. The

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Department of Pathology ("Sotiria" Chest Diseases Hospital of Athens, Greece) and the local ethics committee gave permission to use those tissues for research purposes. Written informed consent was obtained from each patient in line with 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 of the specimens derived from local or extended lung surgical resections (lobectomies). All corresponding hematoxylin and eosin (H&E)-stained slides were reviewed by two pathologists for confirmation of diagnosis and classification according to World Health Organization (WHO 2000) grading criteria. Clinicopathological data (socio-demographic characteristics and medical history of the study participants) are demonstrated in Table 1.

Immunohistochemistry

Ready-to-use rabbit polyclonal anti-PTEN antibody (PN37-InVitrogen/Zymed, San Francisco, USA) was used. IHC for PTEN antigen was carried out on 3 μm serial sections of the tissue blocks. Two slides were deparaffinized and rehydrated. Both of them were enzyme-digested (proteinase K) for 10 min at 37º C. The NBA kit (Zymed/InVitrogen, San Francisco, USA) was used for the next detection steps. Blocking solution was applied to the slides for 10 min, followed by 1h incubation using the antibodies (dilution 1:20 and 1:60, respectively) at room temperature. Following incubation with the secondary antibody for 10 min, diaminobenzidine-tetrahydrochloride (DAB) 0.03% containing 0.1% hydrogen peroxide was applied as chromogen and incubated for 5 min. Sections were counterstained, dehydrated and cover-slipped. IHC protocol was performed using an automated staining system (I 6000 - Biogenex, San Ramon, CA, USA). Diffuse cytoplasmic staining was observed regarding PTEN expression (Figure 1). For negative control slides, the primary antibody was omitted. Breast cancer tissue sections expressing PTEN protein and normal-appearing lung epithelia were used as control staining pattern. Protein expression levels were evaluated quantitatively using an image analysis macro (Figure 2).

Computerized image analysis assay (CIA)

PTEN protein expression levels were evaluated quantitatively by estimating the staining intensity levels. We performed CIA using a semi-automated system (Microscope CX-31, Olympus, Melville, NY, USA, Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan). Areas of interest were identified (10 fields of each immunostained case at ×40) and filed in a digital

Table 1. Clinical data and PTEN IHC analysis

Variables	*PTEN expression**			*p-value*
	Negative (0) (N=24)	*Low (1+) (N=29)*	*Moderate/High (2+/3+) (N=8)*	
Age (years) (mean±SD**)	63.29 ± 7.93	64.34 ± 8.20	57.75 ± 13.16	0.18
Gender, N (%)				0.07
Male	17 (70.83)	24 (82.76)	8 (100.00)	
Female	7 (29.17)	5 (17.24)	0 (0.00)	
Tumor size (cm) (mean ±SD)	5.32 ± 2.88	4.57 ± 2.73	5.63 ± 2.94	0.50
Stage				0.18***
I	6 (25.00)	14 (48.28)	4 (50.00)	
II	8 (33.33)	4 (13.79)	2 (25.00)	
III	10 (41.67)	11 (37.93)	2 (25.00)	
N stage, N (%)				0.84
N0	12 (50.00)	16 (55.17)	4 (50.00)	
N1	5 (20.83)	5 (17.24)	2 (25.00)	
N2	7 (29.17)	8 (27.59)	2 (25.00)	
Histologic subtype, N (%)				0.49
Adeno	14 (58.33)	18 (62.07)	3 (37.50)	
Squamous	10 (41.67)	11 (37.93)	5 (62.50)	
Grade, N (%)				0.25
I	14 (58.33)	17 (58.62)	7 (87.50)	
II/III	10 (41.67)	12 (41.38)	1 (12.50)	
Chemotherapy, N (%)				0.88
No	6 (25.00)	6 (20.69)	2 (25.00)	
Yes	18 (75.00)	23 (79.31)	6 (75.00)	
Metastasis (overall), N (%)				0.14
No	12 (50.00)	15 (51.72)	7 (87.50)	
Yes	12 (50.00)	14 (48.28)	1 (12.50)	
Smoking (> 1 pack per day, continuously)	74.42 ± 36.95	72.34 ± 47.64	71.50 ± 37.94	0.98

*Based on image analysis (value range at RGB protocol: 0-255) the groups demonstrated the following staining intensity values: score 0: >178, score 1+: 148-169, score 2+: 125-143, score 3+: 82-119; **SD: standard deviation; ***stage I/II vs. stage III (p=0.05)

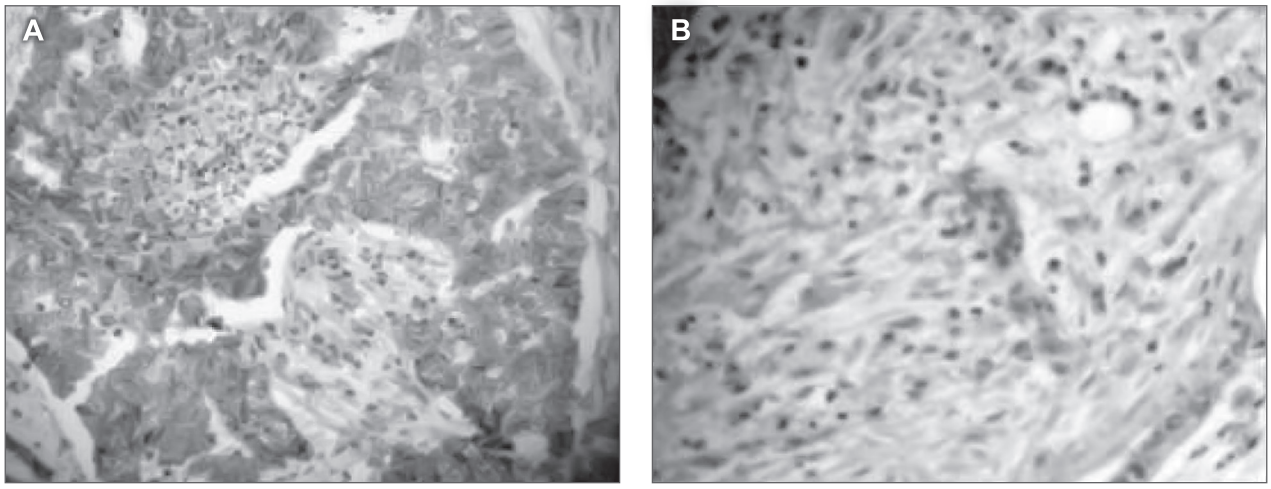

Figure 1. Different PTEN protein expression patterns in NSCLC. **A:** A case of a normal PTEN expression. Note the diffuse cytoplasmic staining pattern. **B:** In contrast, another case showing loss of PTEN expression. Original magnification ×10.

Figure 2. Performance of digital image analysis protocol in evaluating PTEN protein expression in NSCLC cases. At the first step, a digital image is filtered through the system **(A)**, followed by a semi automated analysis **(B)**, and finally a value of staining intensity level is extracted and exported in an excel file **(C)**. Red labelled areas cover the area of PTEN expression in the specific field.

base. A macro was implemented for measuring the amount of PTEN protein expression. Grouping the extracted staining intensity values (value range at RGB protocol: 0-255; values increasing to 255 correlated with loss of expression, whereas values decreasing to 0 demonstrated overexpression of the molecule), we considered complete absence of PTEN stain as 0 score, moderate expression as 1+, and moderate/strong expression as 2+/3+ score. Values in groups and cut-offs based on digital analysis are demonstrated in Table 1.

Statistical analysis

Continuous data are presented as mean ± standard deviation, whereas categorical data as absolute and relative frequency. Several variables were examined including age, gender, tumor size, stage, lymph node involvement, histologic subtype, grade, chemotherapy administration, occurrence of metastasis, and smoking. Student's t-test for continuous data in two independent samples, ANOVA test for continuous data in more than two independent samples, x^2 test for categorical data and Fisher's exact test for categorical data with limited number of frequencies were carried out. The significance level was set at p=0.05. The SAS statistical package (Version 9.1, SAS Institute Inc, Cary, NC) was used for data analysis.

Results

Of 61 patients 49 (80.33%) were male and 12 female (19.67%), with a mean age of 63.07±8.96 years (range 39-78). Twenty-four (39.34%) patients were classified as stage I, 14 (22.95%) as stage II and 23 (37.71%) as stage III. Thirty-five (57.38%) patients had adenocarcinoma and 26 (42.62%) squamous cell carcinoma.

Twenty-four (39.34%) tissue samples were characterized by loss of PTEN expression, 29 (47.54%) had low PTEN expression and 8 (13.12%) had high PTEN expression. No significant differences were found in relation to expression of PTEN with age (p=0.18), gender (p=0.07), tumor size (p=0.50), lymph node involvement (p=0.84), histologic subtype (p=0.49), tumor grade (p=0.25), chemotherapy administration (p=0.88), and smoking (p=0.98) (Table 1). Interestingly, patients with progressive loss of PTEN expression seemed to develop metastasis more frequently as compared to patients with PTEN overexpression (stage I/II vs. III, p=0.05). Among the 12 patients with loss of PTEN expression that developed metastasis, 8 (66.67%) were classified as stage III. In the group of 14 patients with low PTEN expression who developed metastasis, 9 (64.29%) had stage III disease. The only patient with PTEN overexpression who developed metastasis had stage III disease and low grade tumor. Furthermore, we attempted an alternative approach, by merging 2 of the 3 levels of PTEN expression, but the results were similar with the previous ones. Specifically, those with high PTEN expression as compared to those with no/low PTEN expression had significantly lower risk to develop metastasis (OR: 0.1; 95% CI: 0.1-1.0; p=0.05).

Discussion

Deregulation of signalling pathways in NSCLC seem to play a critical role for the progression of the carcinogenetic process and also for the prognosis in those patients [9]. Concerning HER2/PI3K/AKT pathway, altered PTEN expression influenced dramatically the response rates of targeted therapeutic approaches based on monoclonal antibodies, such as trastuzumab, especially in breast cancer patients [10,11]. These agents affect negatively the binding of molecules on the specific receptors' extracellular domains, preventing a cascade of events that lead to an abnormal overactivation of the nucleus [12]. In breast cancer patients, reduced or loss of PTEN protein expression levels are responsible for poor response to trastuzumab-based therapy due to activation of PI3K/AKT/mTOR oncoproteins [5,13]. In such patients, although IHC and molecular criteria based on *in situ* hybridization techniques (2+/3+ protein expression score combined with gene amplification) are fulfilled for applying the agent, PTEN downregulation due to point mutations or allelic imbalances does not prevent the abnormal signal transduction to the nucleus [5].

In the current study we investigated the role of PTEN expression in NSCLC. According to several studies there is sufficient association between PTEN expression and the clinical behavior of lung cancer [14,15]. There are many reports where loss of PTEN expression is related with invasion, metastasis and with shorter survival in NSCLC patients [16,17]. Although other studies with inconsistence reports claiming that PTEN expression is not associated with prognosis in patients with lung cancer, 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 PTEN gene are rare in NSCLC but loss of PTEN protein is not an uncommon event [18].

Analyzing the PTEN protein in our sample, we found loss or low PTEN expression in 86.88% of the patients, while PTEN overexpression was associated with lower risk of developing metastases (p=0.05). Concerning lung cancer patients it seems necessary a different therapeutic approach if HER2 molecular criteria are fit with those that have been already reported concerning breast cancer. 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 [19]. On the basis of preclinical data generated in mouse and rat models, a therapeutic window for PI3K inhibitors exists [20]. Preclinical studies of LY294002, a PI3K inhibitor, have indicated that the agent enhances the sensitivity of NSCLC cells to chemotherapy and radiation [21].

In this study we performed a digital-based image analysis in order to estimate quantitatively the PTEN expression levels. Using a modified software macro, we

detected accurately and rapidly the protein staining intensity. Based on the control group (normal-appearing lung epithelia) we determined cut-offs regarding the 4 main groups of staining intensity: loss of expression, low expression, moderate expression and high expression levels. This is an improved and more sophisticated process compared to the conventional eye-microscopy evaluation, because human eye cannot discriminate more than 199 levels of grey, whereas RGB pixel-based digital analysis provides up to 256 such levels [25]. There is an increasing rate of medical publications that support the usefulness and accuracy of this procedure in pathology/cytology slide analyses [22-28].

In conclusion, PTEN deregulation correlates with an aggressive phenotype (involvement in developing metastasis) in NSCLC and this is a significant genetic event for applying a rational targeted therapeutic regimen in such patients, especially in conjunction with HER2/PI3K inhibition.

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