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ORIGINAL ARTICLE

Tumor characteristics, expressions of ERCC1, Bax, p53, IGF1R, Bcl2, Bcl2/Bax and prognostic factors for overall survival in patients with lung carcinoid

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Summary

Purpose: *Neuroendocrine lung tumors (NET) include typical carcinoids (TC), atypical carcinoids (AC), large cell NE carcinoma (LCNEC) and small-cell carcinoma (SCLC), with different clinicopathological profiles and relative grades of malignancy. Although differences between carcinoids and high grade carcinomas are recognized, precise differences and behavior of TC and AC have not been clearly defined. The aim of this study was to better define the differences in the clinical behavior of TC and AC, and to establish new prognostic factors of overall survival (OS), by determining the levels of genetic expression of IGF1R, ERCC1, Bax, p53, Bcl2 and Bcl2/Bax ratio.*

Methods: *The histopathological diagnosis of 52 surgically resected pulmonary carcinoid tumors was made according to the WHO classification. Gene expressions were evaluated by quantitative real-time PCR.*

Results: *The confirmed prognostic factors for overall survival (OS) were pTNM T ($p<0.01$), pTNM N ($p<0.05$), clinical stage ($p<0.05$), type of surgery ($p<0.01$) and histopathological (HP) tumor type ($p<0.05$). Bcl2 mRNA level and Bcl2/Bax ratio were found to have a potential for discrimination of the HP type of tumor (AC vs TC, Receiver Operating Characteristics (ROC) cut-off values 0.1451 and 0.3015, respectively), but without statistically significant impact on OS.*

Conclusions: *In patients with NETs, smaller primary tumor, absence of positive lymph nodes, and TC type of tumor predicted longer OS. Type of resection has influence on OS. Bcl2 expression and Bcl2/Bax ratio might be valuable as independent diagnostic parametars in lung carcinoids. Therapeutic approaches using attenuation of Bcl2 or upregulation of Bax might prove useful in lung NETs.*

Key words: *Bcl2, Bcl2/Bax ratio, lung carcinoid, overall survival*

Introduction

Neuroendocrine tumors (NETs) are known to be a heterogeneous group of tumors that arise from neuroendocrine cells in the body. Lung NETs originate from pulmonary neuroendocrine cells (PNECs) that can be found as individual cells or small PNEC clusters [1,2]. Lung NETs are classified in two groups as (1) well differentiated (low-grade typical carcinoids (TC) and intermediate-grade atypical carcinoids (AC)) and (2) poorly differentiated (high-grade large cell neuroendocrine carcinoma (LCNEC) and small cell lung carcinoma (SCLC)) [3-6]. Well differentiated lung NETs comprise approximately 27% of all NETs and 1-2% of all primary lung cancers [6-8]. Although TC and AC are rare tumors, their prevalence has increased over the last 30 years in both men and women, prob-

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ably due to improvements in detection methods and diagnostics. Unlike other types of lung cancer, the relationship between well differentiated lung NETs and smoking has not been proven yet. Although all NETs share similar morphologic, ultrastuctural and immunohistochemical characteristics, today there is enough evidence that TC and AC can be clearly differentiated from LCNECs and SCLC [2,7,9,10]. Concerning their biological and clinical behavior, TC and AC show a low or intermediate grade of malignancy compared to more aggressive and high grade malignancy LCNEC and SCLC [5,11,12].

At the time of diagnosis, most patients with carcinoids have nonspecific symptoms that often cannot be distinguished from other respiratory conditions (such as asthma or respiratory infections). Well differentiated lung NETs are usualy located centrally in the main or in the lobar bronchi (up to 80% of tumors), with symptoms characteristic for the tumor location (obstructive respiratory symptoms, cough, hemoptysis, dyspnea, wheezing, pneumonitis and chest pain) [2,9,13]. Peripherally located tumors are often asymptomatic. Although TC and AC are considered low or intermediate grade tumors, they can give regional lymph node or distant metastasis (5-20% of TC and 30-40% of AC) which leads to poorer 5-year and 10-year prognosis [7-9,12-14]. Surgical anatomic resection (pneumonectomy or lobectomy) or lung sparing procedures (segmentectomy or sleeve resection) are the treatments of choice in patients with localized TC or AC and are the only curative options [15]. Even after the resection, both TC and AC tend to metastasize 5 up to 10 years after the operation, which is the reason why long term follow up is advised. In patients with advanced and metastatic disease, there are many debates concerning the appropriate treatment algorithms despite numerous options. Thus, it is of great importance to define new prognostic and predictive biomarkers for standard therapy of these tumors [10,16,17].

It has previously been suggested that for lung NETs the rates of proliferation and apoptosis could contribute to their distinctive behavior. Thus, exploring the genetic regulation of apoptosis through evaluation of the tumor suppressor *p53*, and the *Bcl2*/*Bax* balance could be useful to predict the clinical behavior of carcinoids [18]. The observed differences in NETs therapy response might be induced by variations in *ERCC1* levels (Excision repair cross-complementation group 1), which is essential for DNA [19]. Also, as the insulin-like growth factor receptor (IGF1R) plays a role in the induction of the epithelial-mesenchymal transition (EMT), the activation of the IGF1R pathway might be responsible for the occurence of distant metastasis in lung carcinoids [20,21].

In this study, we investigated the relations between characteristics of the patient (age, gender), pathological (pTNM) and clinical staging and HP type, treatment (type of surgery) and levels of gene expressions (*IGF-1R*, *ERCC1*, *Bax*, *p53* and *Bcl2*), as well as the prognostic factors for overall survival (OS) defined as the time from surgery to death from any cause.

Methods

Patients

A total of 52 patients (26 TC and 26 AC) with histopathological diagnosis of primary pulmonary NET treated at the Institute for Pulmonary Diseases in the Clinical Centre of Serbia, were enroled in this study. General characteristics of the patients, primary disease and outcome are presented in Table 1. Only resectable cases were considered and all patients undervent surgical treatment. Informed consent was obtained from all patients and the study was approved by the Ethical Review Board of the Institute for Pulmonary Diseases.

Histology and immunohistochemistry

The histology of all the enrolled patients' samples was reviewed and reclassified by two experts in pulmonary pathology (J.S. and J.M.), and consesus in opinions was present in every case. Formalin-fixed paraffin-embedded blocks (FFPE) were obtained in all cases and tumor tissue sections were processed by routine hematoxylin-eosin (H&E) and immunohistochemical stainings. The diagnosis was made according to criteria based on the 2015 WHO classification of lung carcinoma in which TC and AC are strictly differentiated [3]. Neuroendocrine differentiation of TC and AC was confirmed by Synaptophysin, and ChromograninA cytoplasmic and CD56 membrane stainings. Cellular atypia, punctuate necrosis and mitotic rate 2-10/2mm^2 on H&E samples and Ki-67 proliferative index up to 10 mitoses per 2mm^2 differs TC from AC.

RNA extraction and cDNA synthesis

Total RNA was extracted from 2-5 10 μm thick FFPE tissue sections using RNeasy FFPE Kit (Qiagen, Manchester, UK) utilizing an 18-hour Proteinase K treatment. RNA quantity and purity were assessed spectrophotometrically using BioSpec-nano (Shimadzu Scientific Instruments, Kyoto, Japan). The complementary DNA (cDNA) was prepared using random primers by RT-PCR. 2 μg total RNA were used as a template for MultiScribeTM Reverse Transcriptase (50 U/μL) using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The reaction was conducted in a final volume of 20 μL, using the following program: 25°C for 10 min, 37°C for 120 min, and inactivation at 85°C for 5 min.

Quantitative Real Time PCR (qRT-PCR)

The levels of *Bax* (RefSeq. NM_001291428.1), *Bcl2* (RefSeq. NM_000633.2), T*P53* (RefSeq. NM_000546.5), *ERCC1* (RefSeq. NM_001983.3) and IGF1R (RefSeq. NM_000875.4) mRNA were detected by quantitative real-time PCR (qRT-PCR) using TaqMan® Gene Expression Assays and TaqMan® Gene Expression Master Mix, (Applied Biosystems). PCR reactions were performed on ABI Prism 7500 Sequence Detection System (Applied Biosystems). Non-template controls were included in each amplification. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min followed by 40 cycles of denaturation (15 sec at 95ºC) and annealing/extension (1 min at 60ºC) in a final volume of 20 μL. All the reactions were performed in triplicate, and the fluorescence of the double stranded products was monitored in real time. To exclude variations arising from different inputs of total mRNA to the reaction, gene expression data were normalized to an internal housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, RefSeq. NM_002046.5). Data was analyzed using the classical delta-delta-Ct method, and the results were expressed in relative units.

Statistics

For the normal distribution data testing, the Kolmogorov-Smirnov and Shapiro-Wilk tests were used. Descriptive methods of statistical analysis (frequencies, percentage, mean, median, standard deviation /SD/ and range) were used to summarize the sample data. The statistical significance level was set at α=0.05 and Bonferroni correction was used for multiple testing over the same dataset. For comparison of disease and treatment characteristics among different subgroups, the Kruskal-Wallis, Wilcoxon rank sum, Pearson chi-square and Fisher exact tests were used. The Receiver Operating Characteristics curve (ROC) methods were applied for the investigation of the diagnostic potential of *IGF-1R*, *p53*, *Bax*, *ERCC1*,

Table 1. Patient characteristics; symptoms and signs of disease; primary disease and treatment characteristics; outcome

Characteristics	*n (%)*	*Characteristics*	*n (%)*
Total patients	52 (100)	Localization	
Age (years)		Central	25 (48.08)
Mean (SD)	49.5 (14.55)	Peripheral	27 (51.92)
Median (range)	53 (17-75)	Clinical stage	
Gender		I (Ia+Ib)	27 (51.92)
Male	25 (48.08)	II (IIa+IIb)	17 (32.69)
Female	27 (51.92)	III	8 (15.38)
Endoscopic findings		Type of surgery	
Direct signs	31 (59.62)	Lobectomy, bilobectomy	36 (69.23)
Indirect signs	21 (40.38)	Pneumonectomy	6 (11.54)
Symptoms		Atyp. resection, segmentectomy	10 (19.23)
Cough	46 (88.46)	HP type	
Dyspnoea	24 (46.15)	TC	26 (50.0)
Pain	20 (38.46)	AC	26 (50.0)
Reccurent pneumonia	19 (36.54)	T in pTNM stage	
Haemoptysis	19 (36.54)	T1 (T1a+T1b)	19 (36.54)
Temperature	19 (36.54)	T2 (T2a+T2b)	26 (50.00)
Number of symptoms		T3	7 (13.46)
0 (without symptoms)	3 (5.77)	N in pTNM stage	
1 symptom	2 (3.85)	*LN- (N0)	33 (63.46)
2 symptoms	18 (34.62)	*LN+ (N1+N2)	19 (36.54)
3 symptoms	14 (26.92)	Follow up period (months)	
4 symptoms	8 (15.38)	Mean (SD)	102 (57.94)
5 symptoms	7 (13.46)	Median (range)	93.54 (1.1-236.6)
Symptom duration (months)		Relapse of disease	
No symptoms	3 (5.77)	Without relapse	38 (73.08)
0-3	16 (30.77)	With relapse	14 (26.92)
3-6	9 (17.31)	Outcome	
6-12	7 (13.46)	Alive	38 (73.08)
> 12	17 (32.69)	Dead	14 (26.92)

*Lymph nodes (LN): N0 (without positive lymph nodes), (LN+): N1+N2 (with positive lymph nodes). SD: standard deviation, HP: histopathology, TC: typical carcinoid, AT: atypical carcinoid

Bcl2 and *Bcl2/Bax* for HP type (AC/TC))as well as the prognostic potential for disease outcome (Area Under the ROC curve - AUC ROC according to DeLong's method; Likelihood ratio test for AUC ROC; DeLong test for two correlated ROC curves). The following methods of OS were used: Kaplan-Meier product limit method for graphical presentation; median with corresponding 95% confidence interval (95%CI) for description and Log-rank test. The Cox proportional hazard regression (univariate and multivariate) method was used for the estimation of prognostic factors for OS: Hazard Ratio (HR) with corresponding 95% CI and Likelihood ratio test. The statistical analysis was done in the program R (version 3.3.2 (2016-10-31) - "Sincere Pumpkin Patch"; Copyright (C) 2016 The R Foundation for Statistical Computing; Platform: x86_64-w64-mingw32/x64 (64-bit); downloaded: January 21, 2017).

Results

Patients, disease and therapy characteristics. Symptoms and signs of disease. Outcome

Patient and disease characteristics are presented in Table 1. In the 52 analyzed patients, frequencies of AC and TC tumors were equal (26 pts per group), gender categories were similar and median age was 53 years. The peripherally localized tumors were slightly more frequent compared to centrally localized tumors (51.92% and 48.08% respectively, Table 1). All patients underwent bronchoscopy before surgery and in 31/52 (59.62%) cases direct endoscopic signs of tumor were present.

Table 2. Expression levels of IGF1R, p53, Bax, ERCC1, Bcl2 and Bcl2/Bax ratio with results of correlation analysis for pairs of IGF1R, p53, Bax, ERCC1, Bcl2 and Bcl2/Bax expressions (n=38)

Characteristics	*Mean (SD)*	*Median (range)*
Levels of expression		
IGF1R	0.96 (0.08)	0.95 (0.78-1.14)
p53	1.00 (0.06)	0.99 (0.88-1.17)
Bax	0.94 (0.06)	0.93 (0.81-1.13)
ERCC1	1.04 (0.07)	1.05 (0.86-1.20)
Bcl2	1.16 (1.86)	0.32 (0.001-6.78)
Bcl2/Bax	1.20 (1.95)	0.35 (0.001-7.22)
Correlations	*Spearman's rho**	*p value*
Pairs of expression		
IGF1R and p53	0.573070	<0.01
IGF1R and Bax	0.480906	<0.01
IGF1R and ERCC1	0.5275194	<0.01
IGF1R and Bcl2	0.2896378	ns#
IGF1R and Bcl2/Bax	0.2690666	ns#
p53 and Bax	0.5212015	<0.01
p53 and ERCC1	0.6951907	<0.01
p53 and Bcl2	0.3987525	<0.05
p53 and Bcl2/Bax	0.3722712	<0.05
Bax and ERCC1	0.7089397	<0.01
Bax and Bcl2	0.4953496	<0.01
ERCC1 and Bcl2	0.4594595	<0.01
ERCC1 and Bcl2/Bax	0.4294781	<0.01

* Spearman's rank correlation coefficient rho; ns#: not statistically significant (i.e. p>0.05)

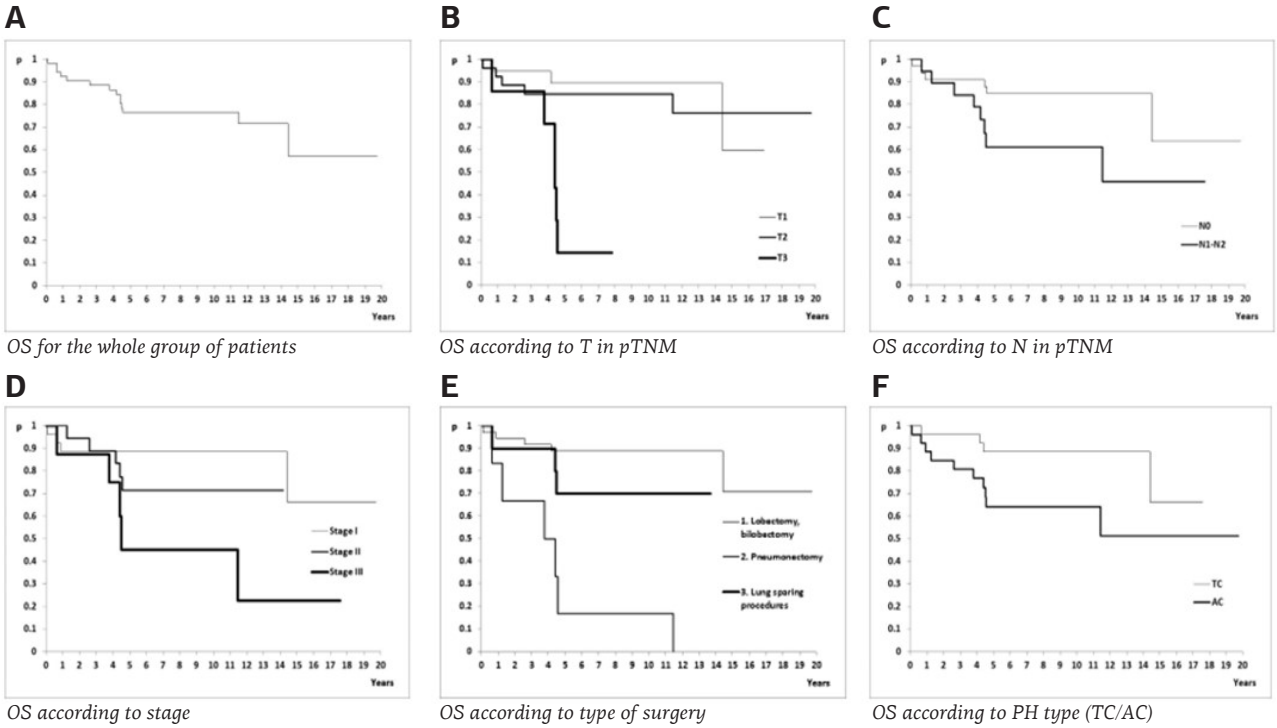


Figure 1. Kaplan-Meier plots for overall survival (OS): **(A)** Whole group and according to: **(B)** T in pTNM (p<0.01); **(C)** N in pTNM (p<0.05); **(D)** tumor stage (p<0.05); **(E)** type of surgery (p<0.01); **(F)** HP type (p<0.05).

Table 3. General characteristics of patients, primary disease, treatment and outcome according to tumor HP type (TC vs AC)

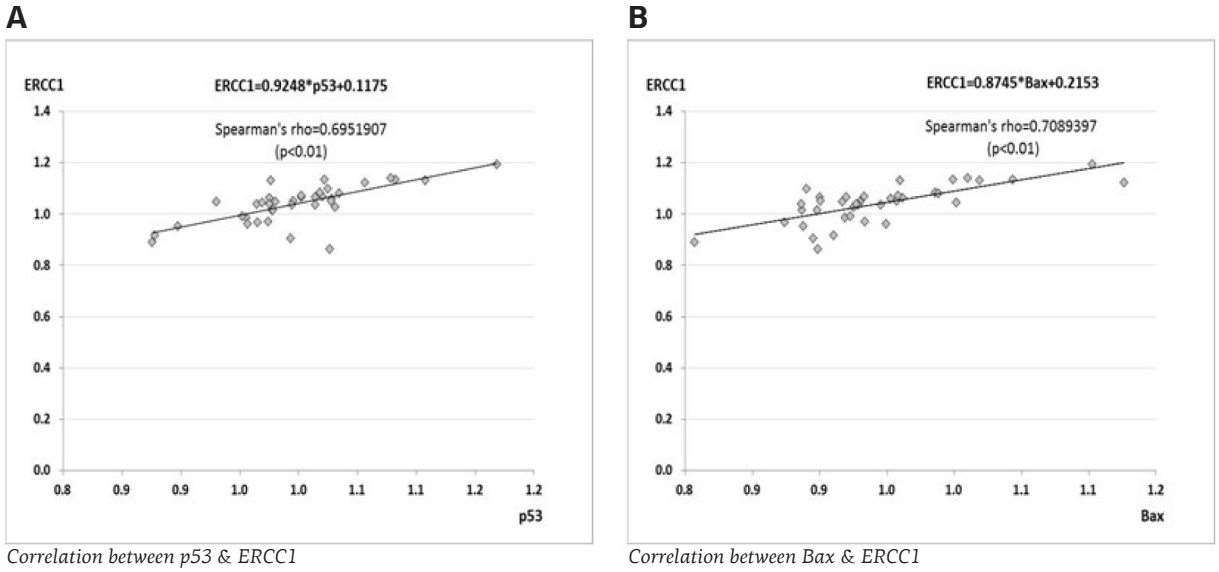
Characteristics (n=52)	*n (%)*		*Fisher exact Test*
	TC	AC	
Age (years)			Wilcoxon rank sum
Mean (SD)	46.65 (17.45)	52.35 (10.50)	ns*
Median (range)	49.5 (17-75)	53 (29-72)	
Gender			Pearson Chi-square
Male	11 (42.31)	14 (53.85)	ns*
Female	15 (57.69)	12 (46.15)	
Localization			Pearson Chi-square
Central	11 (42.31)	14 (53.85)	ns*
Peripheral	15 (57.69)	12 (46.15)	
T in pTNM stage			p <0.01
T1 (T1a+T1b)	14 (53.85)	5 (19.23)	
T2 (T2a+T2b)	11 (42.31)	15 (57.69)	
T3	1 (3.85)	6 (23.08)	
N in pTNM stage			p <0.05
#Ln-(N0)	21 (80.77)	12 (46.15)	
#Ln+(N1+N2)	5 (19.23)	14 (53.85)	
Clinical stage			p <0.05
I (Ia+Ib)	18 (69.23)	9 (34.61)	
II (IIa+IIb)	7(26.93)	10 (38.46)	
III	1 (3.85)	7 (26.92)	
Type of surgery			ns*
Lobectomy, bilobectomy	21 (80.77)	15 (57.69)	
Pneumonectomy	1 (3.85)	5 (19.23)	
Segmentectomy or atyp.resection	4 (15.38)	6 (23.08)	
Relapse of disease			ns*
Without relapse	22 (84.62)	16 (61.54)	
With relapse	4 (15.38)	10 (38.46)	
Total pts	26 (100)	26 (100)	-
Expressions (n=38)	*TC*	*AC*	*Wilcoxon rank sum*
IGF1R			ns*
Mean (SD)	0.95 (0.09)	0.96 (0.08)	
Median (range)	0.94 (0.78-1.14)	0.97 (0.78-1.20)	
p53			ns*
Mean (SD)	1.00 (0.07)	0.99 (0.04)	
Median (range)	1.01 (0.88-1.17)	0.99 (0.88-1.08)	
Bax			ns*
Mean (SD)	0.95 (0.06)	0.94 (0.07)	
Median (range)	0.93 (0.89-1.10)	0.93 (0.81-1.13)	
ERCC1			ns*
Mean (SD)	1.06 (0.07)	1.02 (0.08)	
Median (range)	1.06 (0.92-1.20)	1.04 (0.86-1.14)	
Bcl2			ns*
Mean (SD)	1.81 (2.43)	0.50 (0.53)	
Median (range)	0.26 (0.01-6.78)	0.39 (0.001-2.29)	
Bcl2/Bax			ns*
Mean (SD)	1.88 (2.55)	0.53 (0.57)	
Median (range)	0.28 (0.01-7.22)	0.42 (0.001-2.55)	
Total pts	19 (100%)	19 (100%)	-

#Lymph nodes: ns*: not statistically significant (i.e. p>0.05)

Table 4. Results of the ROC analysis for IGF1R, p53, Bax, ERCC 1, Bcl2 and Bcl2/Bax ratio

Levels of expression	*PH type (AC/TC)*		*Disease outcome (dead/alive)*	
	AUC ROC (95%CI)*	Test**	AUC ROC* (95%CI)	Test**
IGF1R	57.9% (38.9-76.8%)	ns#	47.0% (27.3-66.8%)	ns#
p53	57.8% (38.8-76.6%)	ns#	57.5% (37.7-77.3%)	ns#
Bax	54.0% (35.1-72.9%)	ns#	48.9% (27.5-70.3%)	ns#
ERCC 1	62.9% (44.7-81%)	ns#	56.3% (36.3-76.2%)	ns#
Bcl2	47.9% (27.5-68.2%)	p<0.01	53.8% (32.4-75.2%)	ns#
Bcl2/Bax	49.0% (28.6-69.4%)	p<0.01	54.4% (32.9-75.9%)	ns#

*Area under the ROC curve (DeLong's method); **Likelihood ratio test for AUC ROC; #ns: not statistically significant (p≥0.05)



Correlation between p53 & ERCC1 *Correlation between Bax & ERCC1*

Figure 2. Linear regression as a model for correlation between: **(A)** p53 & ERCC1; **(B)** Bax & ERCC1.

Table 5. Results of the ROC analysis for Bcl2 and Bcl2/Bax in regard to PH type (AC vs TC)

Characteristics	*Bcl2*	*Bcl2/Bax*
AUC ROC* (95%CI)	47.9% (27.5-68.2%)	49.0% (28.6-69.4%)
Likelihood ratio test**	p <0.01	p <0.01
ROC-cut-off value#	0.1451	0.3015
Sensitivity (95% CI)	89.5% (73.7-100.0%)	68.4% (47.4-89.5%)
Specificity (95% CI)	36.8% 15.8-57.9%)	57.9% (36.8-79.0%)

*Area Under the ROC curve (DeLong's method); **Likelihood ratio test for AUC ROC; #Levels of expression with maximum sensitivity and specificity

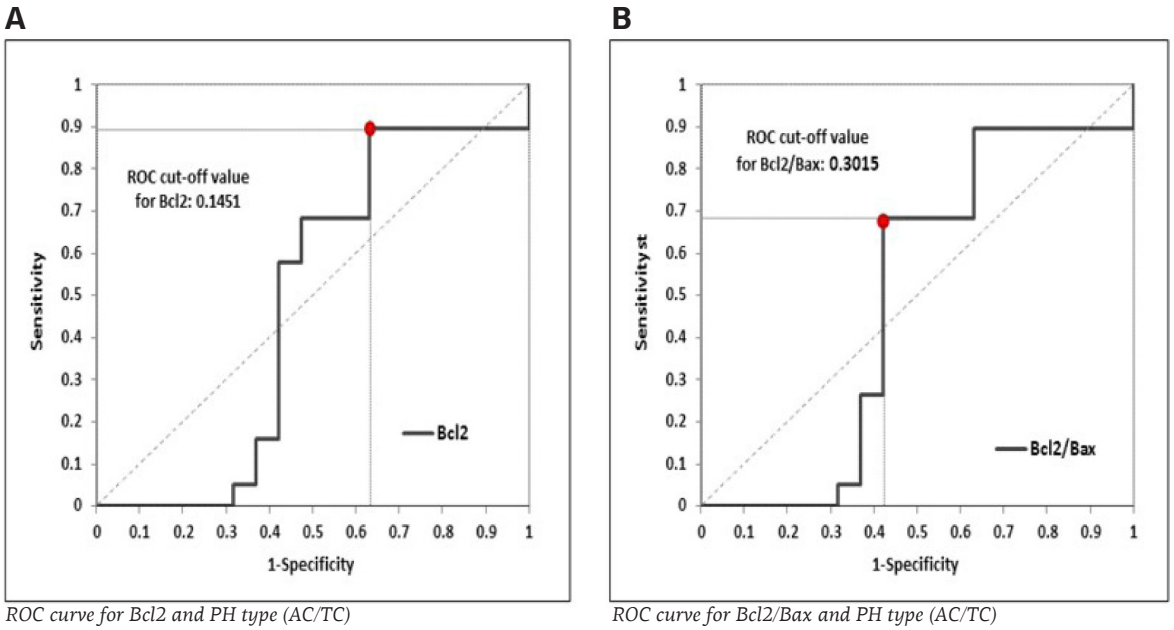
Cough was the most common symptom (88.46%) in comparison with others (dyspnoea, pain, haemoptysis, recurrent infection and temperature). Only 3/52 (5.77%) patients were asymphomatic while all other patients (94.23%) had at least one presenting symptom at the time of diagnosis. Most often patients initialy had two or three symptoms (61.54%; Table 1) and they were present either for more than one year prior to diagnosis (32.69%, Table 1), or very shortly for up to 3 months prior to diagnosis (30.77%). All patients were treated operatively. Lobectomy or bilobectomy were performed in most cases (69.23%).

After histopathological and immunohistochemical analyses, according to the 7th pTNM classification, the most common categories were T2 (T2a+T2b) (50%; Table 1) and N0 status (63.46%; Table 1), as well as stage I (Ia+IB) (51.92%; Table 1).

During the follow-up period with a median of 93.54 months and range of 1-237 months (Table 1), a total of 14/52 patients (26.92%) experienced relapse and then died. The median OS was not reached and the 5-year survival rate was 76.5% (Figure 1-case A).

Comparison between TC and AC

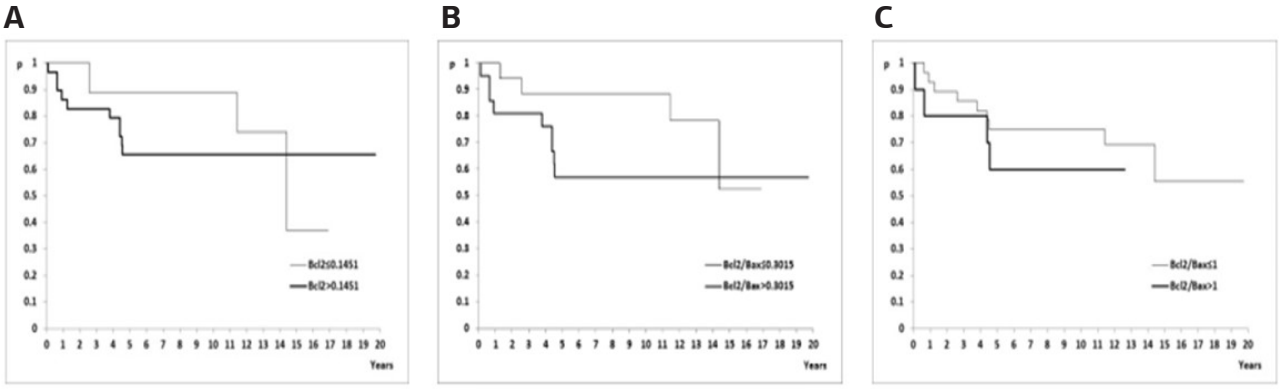
Investigation of all characteristics between TC and AC group showed that patients with AC had more frequently: (1) direct endoscopic signs of tumor, but only haemoptysis with statistically



ROC curve for Bcl2 and PH type (AC/TC)

ROC curve for Bcl2/Bax and PH type (AC/TC)

Figure 3. ROC curves for **(A)** Bcl2, **(B)** Bcl2/Bax ratio for the HP type of tumor (AC/TC).



OS according to ROC cut-off value for Bcl2

OS according to ROC cut-off value for Bcl2/Bax

OS according to a validated reference cut-off value for Bcl2/Bax ratio

Figure 4. Overall survival (OS) according to ROC cut-off values for **(A)** Bcl2 (p>0.05); **(B)** Bcl2/Bax (p>0.05); and **(C)** according to a validated reference cut-off value for Bcl2/Bax ratio (p>0.05) [18].

significant difference (TC vs AC: 5/26 (19.23%) vs 14/26 (53.85%); Pearson Chi square Test: x^2_1=6.72; p=9.55*10^{-3}); (2) bigger tumors (higher T stage; p<0.01, Table 3); (3) positive lymph nodes (N1+N2; p<0.01; Table 3); (4) higher tumor stage (II/III; p<0.01; Table 3); and (5) disease relapse but without statistically significant difference (Table 3).

Levels of expression of IGF1R, p53, Bax, ERCC1, Bcl2, Bcl2/Bax ratio and their correlations

Gene expression analysis was successful in 38/52 (73.08%) patients, and the obtained results are shown in Table 2. Correlation analysis between pairs of expressions without mutually dependent pairs like *Bcl2* and *Bcl2/Bax*; *Bax* and *Bcl2/Bax* confirmed relatively strong positive correlations (Spearman's rho near to 0.7) between *p53* & *ERCC1* and *Bax* & *ERCC1* (p<0.01 in both cases (Table 2 and Figure 2-cases A and B).

There was no statistically significant difference between the levels of expression of IGF1R, *p53*, *BAX*, ERCC 1, *Bcl2* and *Bcl2* between TC and AC groups (Table 3).

Discriminative potential of IGF1R, p53, Bax, ERCC 1, Bcl2 levels of expression and the Bcl2/Bax ratio for the HP type of tumor (AC/TC) and disease outcome (dead/alive)

Applying ROC analysis, we examined the discriminative potential of gene expressions for the HP type of tumor (AC/TC) and disease outcome (dead/alive). The results confirmed that only *Bcl2* level and *Bcl2/Bax* ratio had a discriminative potential for predicting the HP type of tumor (AC vs TC;

Table 4; Figure 3) with ROC cut-off values 0.1451 for *Bcl2* and 0.3015 for *Bcl2/Bax* (Table 5; Figure 3). The difference between the discriminative potential of *Bcl2* and *Bcl2/Bax* expressions for the AC/TC HP type of tumor was not confirmed (DeLong test for two correlated ROC curves; p=0.2254).

Prognostic factors for overall survival

Survival analysis confirmed that some clinicopathologic characteristics had impact on OS (Table 6). Shorter OS was observed in patients with T3 stage (compared to T1 and T2 stage; p<0.01; Table7; Figure 1 -case B.) and nodal involvement (N1+N2) (compared with N0; p<0.01; Table 7; Figure 1 -case C). Also, patients with stage III had a statistically significant shorter OS compared with patients with stage I (p=0.01; Table 7; Figure 1 -case D). Patients with pneumonectomy as a type of operation had worse OS compared to patients with lobectomy or bilobectomy, but better OS compared to patients with lung-sparing procedures (p=0.01 for both cases; Table 7; Figure 1 -case E). Patients with AC had shorter OS compared to TC (p=0.02; Table 6; Figure 1 - case F).

No statistically significant difference in OS was observed between the categories *Bcl2* and *Bcl2/Bax* neither regarding the determined ROC *cut-off* values (Table 6; Figure 4 - cases A&B respectively), nor regarding the previously validated cut-off value 1 [18] (Table 6; Figure 4 - case C). Median OS was

Table 6. Results of the subgroup survival analysis

Characteristics	*Subgroup survival analysis*		
	n (%)	*Median OS with 95% CI (months)*	Log-rank test
Whole group of patients	*52 (100)*	*NR#*	
Gender			ns*
Male	25 (48.08)	NR#	
Female	27 (51.92)	NR#	
T in pTNM stage			p<0.01
T1 (T1a+T1b)	19 (36.54)	NR#	
T2 (T2a+T2b)	26 (50.00)	NR#	
T3	7 (13.46)	52.8 (>45.3)	
N in pTNM stage			p<0.05
Ln-(N0)	33 (63.46)	NR#	
Ln+(N1+N2)	19 (36.54)	137 (>52.8)	
Clinical stage			p<0.05
I (Ia+Ib)	27 (51.92)	NR#	
II (IIa+IIb)	17 (32.69)	NR#	
III	8 (15.38)	54.1 (>52.8)	
Type of surgery			p<0.01
Lobectomy, bilobectomy	36 (69.23)	NR#	
Pneumonectomy	6 (11.54)	49.1 (>15)	
Segmentectomy or atypical resection	10 (19.23)	NR#	
HP type			p<0.05
TC	26 (50.0)	NR#	
AC	26 (50.0)	NR#	
Patients with expressions	*38 (100)*	*NR#*	-
Bcl2 (ROC cut-off)			ns*
≤0.1451	9 (23.68)	173 (>137)	
>0.1451	29 (76.32)	NR#	
Bcl2/Bax (ROC cut-off)			ns*
≤0.3015	28 (73.68)	NR#	
>0.3015	10 (26.32)	NR#	
Bcl2/Bax**			ns*
≤1	17 (44.74)	NR#	
>1	21 (55.26)	NR#	

ns*: not statistically significant (p>0.05); NR#: not reached; Bcl2/Bax** [18]

Table 7. Survival analysis results of category pairs testing

Pairs	*Log-rank test*
T in pTNM	
T1 vs T2	p*=0.6793
T1 vs T3	p*=0.0002
T2 vs T3	p*=0.0007
Stage	
I (Ia+Ib) vs II (IIa+IIb)	p*=0.2129
I (Ia+Ib) vs III	p*=0.0102
II (IIa+IIb) vs III	p*=0.0989
Type of surgery	
Lobectomy vs pneumonectomy	p*=4.3×10^{-7}
Lobectomy vs segmentect or atypical resection	p*=0.1725
Pneumonectomy vs segmentectomy or atypical	p*=0.0111

* According to Bonferroni correction (p<0.05/3=0.0167)

worse in the group with a higher *Bcl2*/*Bax* ratio (82.4 months vs 137.4 months), but without statistically significant difference (p= 0.15).

Discussion

NE tumors exibit a spectrum of histologies, clinical profiles and biologic behavior ranging from relatively indolent TC to biologically aggresive tumors such is SCLC. High grade neuroendocrine tumors (LCNEC and SCLC) have worse prognosis with a clear correlation to the clinical stage [6,12,14,22]. However, there are still no definitive conclusions when distinguishing TC from AC considering their diagnosis, biological behavior, prognosis and therapeutic algorithms.

Some studies showed that more aggressive tumors tend to affect older male patients [10,13,23]. However, in this study, no significant difference in gender or age distribution between TC and AC was observed, although median age was higher in AC than in TC (53 vs 49.5 years, respectively) and male patients more frequently had AC tumors than females (54% vs 46%). Patients with NET usually present with nonspecific symptoms which lead to a delay in diagnosis. In both groups (TC and AC) most frequent presenting symptoms were cough and pneumonitis. Literature data shows that in up to 50% of NET cases reported up to 2-year long periods from the occurence of a first symptom to diagnosis of lung NET [24-26]. This study emphasized the importance of recognizing presenting symptoms, especially in the case of the more agressive AC. Patients with AC had a shorter time from onset of symptoms to the time of diagnosis, probably due to more frequent central localization of the tumor (53.85%).

Analyzing the differences between AC and TC tumors, most previous studies showed that AC had higher rates of lymph node involvement at diagnosis than TC, which was associated with poorer 5- and 10-year survival rates [7,14,27,28]. In this study, we detected that patients with AC more frequently had higher T stage and positive nodal involvement at the time of diagnosis (p=0.02), confirming the importance of nodal involvement for the prognosis of both TC and AC, as it was connected to worse prognosis and tendency to relapse. As much as 63.46% of our patients had pN0 stage, which was connected to longer median OS compared to patients with pN2 stage.

Molecules important for cell proliferation, growth, angiogenesis and metastatic spread have previously been explored as potential therapeutic targets in NETs [29]. As there are no definitive conclusions when distinguishing TC from AC, we performed a study that aimed to evaluate the significance of expression levels of *p53*, *Bax*, *Bcl2*, *ERCC 1* and *IGF1R* on tumor malignant potential.

It has been reported that the expression of *Bcl2* and *Bax* changes with increasing NET malignancy [30]. Moreover, *Bcl2* overexpression, *Bax* down-regulation, and *Bcl2*/*Bax* ratio >1 correlated with a statistically shorter survival in lung NETs [18]. Some studies showed that greater aggressiveness was associated with *Bcl2* overexpression [31] and that high level of *Bax* expression correlated with longer survival which lead to the conclusion that carcinoids had a better prognosis compared to more aggressive NETs such as LCNET and SCLC [18,32,33]. In our study, higher levels of *Bax* were measured in TC patients compared to AC patients. Also, patients with higher median levels of *Bcl2* had an AC HP type, leading to a higher *Bcl2*/*Bax* ratio in patients with AC HP type. These data reinforce the assumption that the fine balance between apoptosis-related molecules plays a vital role in tumorigenesis. ROC analysis confirmed that *Bcl2* level and *Bcl2*/*Bax* ratio have a discriminative potential for predicting the HP type of tumor (AC vs TC). A high correlation of expressions between *p53* & *ERCC 1* and *Bax* & *ERCC 1* (p<0.01) was detected, so a more detailed study of their interaction and roles in lung NET pathogenesis is strongly recommended.

The use of the current pTNM classification proved useful when correlating pTNM stage and OS. As reported in previous studies, patients with AC had worse prognosis compared to TC, which in combination with additional nodal involvement, larger tumor size and higher stage suggests a pos-

sible benefit of adjuvant chemotherapy [34]. Better OS was detected in patients who undervent lobectomy or bilobectomy in comparison to pneumonectomy or lung-sparing procedures (segmentectomy or atypical resection). Although the median OS was worse in the group with a higher *Bcl2*/*Bax* ratio, there was no statistically significant difference, neither using the determined ROC cut-off values nor using the previously validated cut-off value [18]. The complex role of the dysregulated expression of the *Bcl2* family members in survival of lung NET patients is yet to be elucidated and warrants more extensive studies on larger cohorts of patients..

Conclusions

In properly selected patients with lung NETs the type of resection increases OS. Also, smaller primary tumor, absence of positive lymph nodes, smaller stage and TC type of tumor predict longer OS. *Bcl2* expression and *Bcl2*/*Bax* ratio might be valuable as independent diagnostic parameters for the AC vs TC types in lung carcinoids. As the HP type of carcinoids (AC/TC) has an impact on OS, research on a larger sample size could also examine the prognostic potential of *Bcl2* and *Bcl2*/*Bax* for OS. Therapeutic approaches using attenuation of *Bcl2* or upregulation of *Bax* might prove useful in lung NETs.

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Conflict of interests

The authors declare no conflict of interests.

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