Role of *Bcl-2*, *Bax*, and *Bak* in Spontaneous Apoptosis and Proliferation in Neuroendocrine Lung Tumors: Immunohistochemical Study

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Fifty-six primary neuroendocrine lung tumors were examined morphologically and histologically and their apoptosis level was determined. Malignant carcinomas were characterized by increased apoptotic index and enhanced expression of *Bcl-2*, *Bak*, *p53*, and *Ki-67* compared to typical carcinoid. However, apoptosis in these tumors was not completed. Proteins of the *Bcl* family play an important role in the regulation of spontaneous apoptosis in neuroendocrine lung tumors. *Bcl-2* accumulating in the nucleus is a morphological analogue of phosphorylated inactive form of this protein, which does not inhibit apoptosis. Expression of *Bcl-2* and *Bax* decreases in small-cell lung carcinoma (SCLC) with metastases indicating attenuation of apoptosis and development of metastatic clones resistant to apoptosis induces.

Key words: *small-cell lung carcinoma; typical carcinoid; malignant carcinoid; apoptosis; biomolecular markers*

Bcl-system including oncoproteins affecting apoptosis (*Bax*, *Bad*, *Bid*, *Bak*) and proliferation (*Bcl-2*, *Bcl-xL*, *Raf*) is a key factor regulating these processes. Genes of the *Bcl* family can be activated by various factors including *p53* protein. They are localized on mitochondrial membranes and control proliferation and apoptosis by modulating mitochondrial membrane permeability and cytochrome *c* release to the cytoplasm. This regulation is mediated by competitive binding of apoptotic and antiapoptotic *Bcl* proteins forming homo- and heterodimers due to the presence of homologous domains in the structure of these proteins. The interaction of *Bcl-2* — *Bcl-2*, *Bcl-2* — *Bcl-xL*, and *Bcl-2* — *Raf* domains with cytochrome *C* induces proliferation, while *Bax* — *Bax*, *Bax* — *Bid*, *Bak* — *Bad* and other domains cause apoptosis [2,6]. Some authors identified *Bcl-2* in endoplasmic reticulum and on karyolemma [3]. Experiments with vinblastine-induced *Bcl-2* phosphorylation in cultured small-cell lung carcinoma (SCLC) Ms-1 showed that phosphorylated inactive *Bcl-2* localized on karyolemma can induce, but not inhibit apoptosis [8].

When studying spontaneous apoptosis in neuroendocrine lung tumors (NELT), we revealed different roles of *Bcl* in this process. We found a direct correlation between *Bcl-2* expression and apoptotic index, which coincides with the data on higher survival of *Bcl-2*-positive SCLC, but contradicts the data of other authors reporting inhibitory effect of this oncoprotein on apoptosis [2].

The purpose of the present study was to examine the role of *Bcl-2*, *Bak*, and *Bax* in induction of spontaneous proliferation and apoptosis in carcinoids and SCLC.

MATERIALS AND METHODS

Complex morphological and immunohistochemical study of 56 primary NELT (42 men and 14 women,

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TABLE 1. Expression of *Bcl-2, Bax, Bak* (Score), *p53, Ki-67*, and Apoptosis in NELT

Parameter	SCLC		Carcinoid	
	classic	combined	malignant	typical
Bcl-2	4.1	3.7	1.6	1.8
Bax	1.9	2.3	0.5	2
Bak	3.5	3.2	1.0	3.3
p53, %	59.7	42.16	30	18.1
Ki-67, %	40.4	45.8	40	20.05
AI, %	5.6	3.4	0.29	0.1

average age 48 years, operated in surgical clinic of I. M. Sechenov Moscow Medical Academy in 1981-1997) was conducted. Histological types of the tumors were determined according to WHO Lung Tumor Classification (1999) [4]. SCLC (28 classic and 12 combined) was diagnosed in 40 cases and carcinoid tumors (11 typical and 5 malignant) were revealed in 16 cases. Carcinoid tumors were more frequent in women (2:1), while SCLC — in men (10:1). Serial paraffin sections were stained with hematoxylin and eosin, alcian blue, and periodic acid—Schiff reaction. Some sections were pretreated in a microwave stove and examined using immunohistochemical technique with avidin-biotin labeling and DAB (ABC kit vector, CA, DAB kit, Dianova). Mono- and polyclonal antibodies against chromogranin (Dako), pancytokeratins (Immunotech), *Ki-67* (Dianova), *Bcl-2* (Dako), *Bak* (Calbiochem), *Bax* (Calbiochem) and *p53* (Dako) were used. Positive and negative controls were run simultaneously. Apoptotic cells were identified with TUNEL-test using Enzo ApopDetek cell death assay system (Enzo Diagnostics). Pyronin G and hematoxylin were used as background dyes. The reaction was scored (chromogranin, pancytokeratins, *c-myc*, *Bcl-2*, *Bak*, *Bax*) or presented as a number of positive cells per 300 tumor cells (*Ki-67*, *p53*, apoptotic index; AI).

RESULTS

NELT were characterized by the absence of phagocytosis of apoptotic bodies, which were localized around detritus foci. This incomplete apoptosis is typical of tumor growth. Detritus can be formed due to autolysis of apoptotic bodies (postapoptotic autolysis). AI reached 4-12.3 and 0.1-9% for classic and combined SCLC, and 0.3 and 0.1% for malignant and typical carcinoid, respectively. Thus, SCLC was characterized by a higher apoptosis level compared to carcinoid.

Bcl-2 was expressed in 29 (73%) and 13 (81%) SCLC and carcinoid specimens, respectively. Both classic and combined SCLC showed enhanced *Bcl-2* expression compared to carcinoid tumors (Table 1). Unlike carcinoid, in SCLC *Bcl-2* accumulated mainly in the nuclei (Fig. 1, *a, b*). *Bax* was revealed in 26 (63%) and 10 (63%) SCLC and carcinoid tumors, respectively. The mean level of *Bax* expression in SCLC insignificantly surpassed that in carcinoid tumors, the lowest *Bax* expression was found in atypical carcinoid tumors (Table 1). *Bax* accumulated both in the cytoplasm and nuclei of tumor cells. In typical carcinoid tumors, *Bax* was found in solitary cells assembled in glandular-like structures (Fig. 1, *c*). Clusters of *Bax*-positive cells were observed in SCLC, some of these cells were highly positive (Fig. 1, *d*). No quantitative correlations between *Bcl-2* and *Bax* expression in different NELT types were found. In each NELT tumor type different noncorrelating levels of *Bcl-2* and *Bax* were found, which agrees with previously reported data [5,7]. Expression of *Bax* was significantly lower than *Bcl-2*, especially in SCLC. In most cases, *Bcl-2/Bax* index was higher or equal to 1. Previous studies also showed high levels of *Bcl-2* expression and *Bcl-2/Bax* index in NELT group, a reverse correlation between *Bax* and *Bcl-2* in some tumors, and a significant inversion of *Bcl-2/Bax* index between carcinoid tumors and NELT [1]. Our data showed that the mean expression of *Bak* in SCLC and typical carcinoid tumors was similar, while in malignant carcinoid tumors it was significantly lower (Table 1). The reaction product accumulated both in the cytoplasm and nuclei of

TABLE 2. Apoptosis (in %) in SCLC with Different Expression of *Bcl* Oncoproteins

Type of SCLC	AI
Bcl-2$^-$/*Bax*$^-$/*Bak*$^-$	7.7
Bcl-2$^-$/*Bax*$^-$	1.1
Bcl-2$^-$/*Bak*$^-$	5.5
Bcl-2$^-$	4.5
Bax$^-$	3.6
Bax$^-$/*Bak*$^-$	2
Bak$^-$	1.8

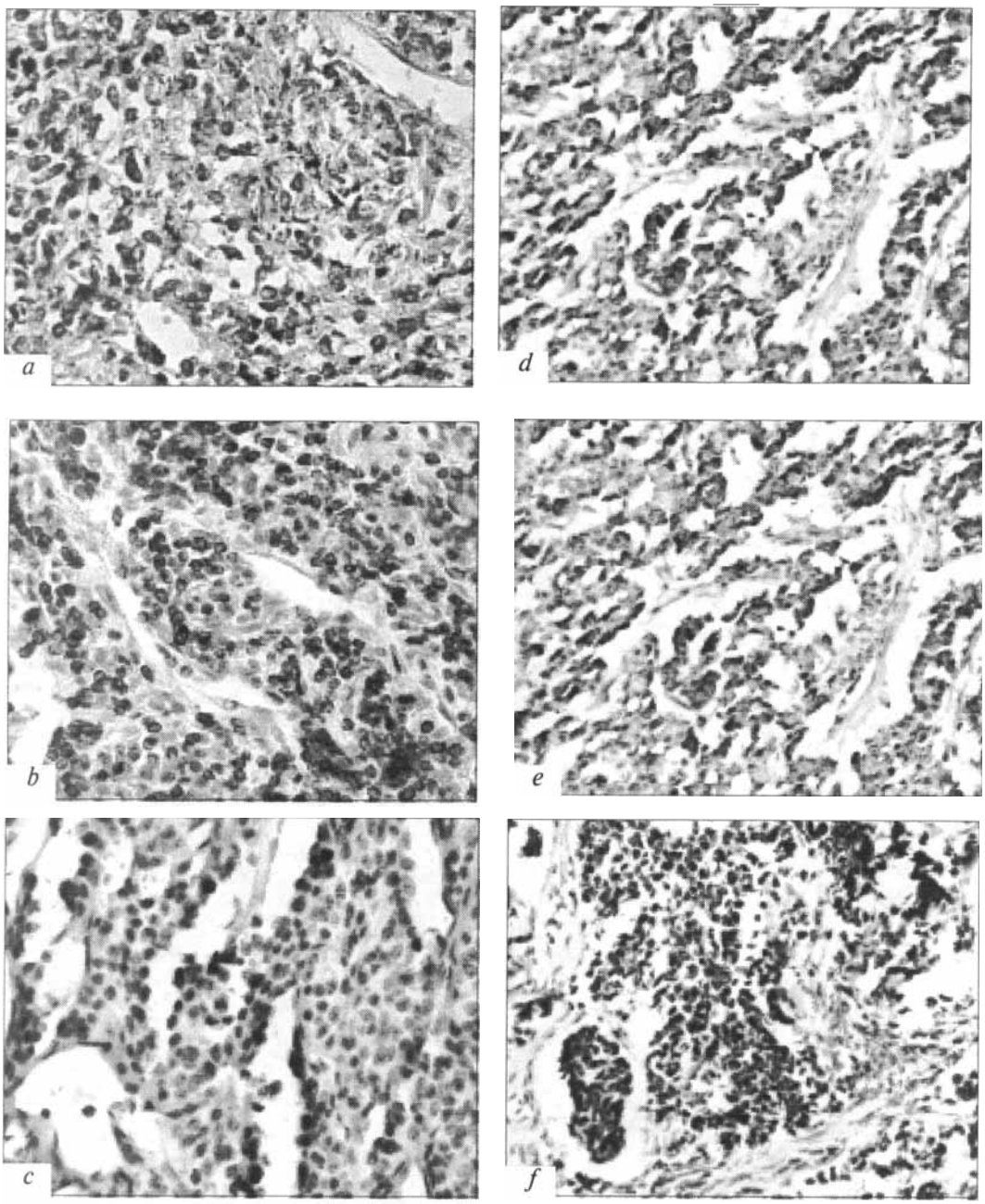

Fig. 1. Accumulation of *Bcl-2* (*a*, *b*), *Bax* (*c*, *d*) and *Bak* (*e*, *f*) in small-cell lung carcinoma (*a*, *b*, *d*, *f*) and typical carcinoid (*c*, *e*). Immunohistochemical reactions, ×00 (*a-d*), ×200 (*e*, *f*).

NELT cells; typical carcinoid was characterized by cytoplasmic localization of *Bak*, while in SCLC *Bak* accumulated both in the cytoplasm and nuclei of tumor cells (Fig. 1, *e*, *f*). In the same NELT *Bak* expression exceeded that of *Bax* and corresponded to *Bcl-2* attaining 6 points; in most cases *Bcl-2/Bak* and *Bax/Bak* indices were equal to 1 and sometimes exceeded it, while *Bax/Bak* index was often below 1. Similar regularities of the expression of these proteins were found in different histological groups of NELT. Qualitative correlation manifested as coexpression of *Bcl* family proteins was found: the absence of *Bcl-2* expression was associated with the abscence of one or two other oncoproteins.

In SCLC *Bcl-2* expression directly correlated with AI: higher apoptosis level corresponded to higher *Bcl-2* expression and *Bcl-2/Bax*>1 (Table 1). This seems to contradict current views, since *Bcl-2* normally inhibits apoptosis. However, SCLC was characterized by nuclear accumulation of *Bcl-2* corresponding to inactive (phosphorylated) form [8], which does not inhibit apoptosis and therefore, it does not affects relatively high level of apoptosis in SCLC. The highest level of apoptosis in SCLC was associated with the absence of

all *Bcl* proteins (Table 2). *Bak* expression correlated both with *Bcl-2* expression and apoptosis level.

As expected, no quantitative correlations between the expression of *Bcl-2*, *Bax*, and *Bak* and the levels of *Ki-67* and *p53* were found, because *Bcl* family proteins do not regulate proliferation directly. However, cytoplasmic *Bcl-2* expression in SCLC to a greater extent was associated with higher *Ki-67* level than nuclear expression of this oncogen.

SCLC without metastases showed higher apoptosis level, high nuclear *Bcl-2* accumulation, and enhanced *Bax* expression compared to SCLC with metastases (Table 3), which confirms our previous data on attenuation of apoptosis in metastasizing SCLC.

Thus, in contrast to typical carcinoid, malignant carcinoid and SCLC were characterized by higher level and incomplete nature of apoptosis. In malignant NELT, the increase of AI was associated with enhanced *Bcl-2*, *Bak*, and *p53* expression and high proliferative activity of tumor cells judging from the expression of *Ki-67*. Proteins of the *Bcl* family play an important role in the regulation of spontaneous apoptosis in NELT due to accumulation of *Bcl-2* in the nuclei, and expression of *Bak* and *Bax*. Since nuclear accumulation of *Bcl* correlates with apoptosis level, it can be regarded as a morphological equivalent of inactive (phosphorylated) form of this protein, which is unable to inhibit apoptosis. Metastasizing SCLC is characterized by decreased expression of *Bcl-2* and *Bax* indicative of attenuation of apoptosis. Progress of SCLC from invasive growth to metastasizing stage is associated with the appearance of a metastatic clone of tumor cells characterized by increased resistance to apoptosis induces.

TABLE 3. Apoptosis and Expression of *p53*, *Ki-67* (in %) and *Bcl* Oncoproteins (Score) in SCLC Depending on the Pre-sence of Metastases

Parameter		SCLC	
		without metastases	with metastases
Apoptosis		4.85	3.48
Expression	*p53*	44.13	61.4
	Ki-67	44.13	45
	Bcl-2	4.5	3.64
	Bax	2.3	1.84
	Bak	3.4	3.36

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