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The Oncology Center of Lublin Province
Department of Human Genetics, Medical University of Lublin
Department of Pathomorphology, Medical University of Lublin
Department of Thoracic Surgery, Medical University of Lublin

BOŻENNA KARCZMAREK-BOROWSKA, AGATA FILIP,
MAŁGORZATA ZDUNEK, ELŻBIETA KOROBOWICZ,
JACEK WOJCIEROWSKI, IWONA KORSZEŃ-PILECKA,
FRANCISZEK FURMANIK

The preliminary estimation of bcl-2, bcl-X_L and p53 genes expression in locally advanced non-small cell lung cancer

Apoptosis (programmed cell death, PCD) constitutes one of main mechanisms that control the delicate balance between cell proliferation and cell death. Any alteration in the process of apoptosis leads to pathological results, e.g. its up-regulation is commonly observed in neurodegenerative disorders, and down-regulation of PCD may contribute to uncontrolled cell proliferation resulting in tumor growth (10). Importantly, it is often connected with decreased susceptibility of such cells to radio- or chemotherapy.

Programmed cell death is a multistep process whose genetic regulation is complex (19). There are many genes whose products act as apoptosis inducers or inhibitors. Some of them also influence other important cell functions, such as cell cycle regulation and genome stability maintenance in case of DNA injury (5). The alterations of these gene expressions are observed in many human malignancies, some of them have proved to be independent diagnostic and prognostic indicators. Until now many genes have been described whose aberrations are involved in the pathogenesis of non-small cell lung cancer. The most important are tumor suppressor genes: *p53*, *Rb*, *cdkn2A (p16)*, *fhit* and protooncogenes belonging to *bcl-2*, *ras* and *myc* families (3,8,12,17,20).

The wild type (wt) *p53* gene acts as 'guardian of the genome' (14). The role of *p53* in cell cycle regulation is that it inhibits G_1/S transition in cells with DNA damage caused by neoplastic transformation or exposure to mutagenic factors (e.g. ionizing radiation) thus disabling the replication of defective DNA. The latest data proves that wtP53 protein is also an important factor in controlling angiogenesis. P53 regulates transcription of the direct inhibitor of angiogenesis, thrombospondin-1, and inhibits the promoter of the *VEGF* gene (13,20). The *p53* gene mutations were described in cases of almost all of human cancers, and the presence of truncated protein product was found in over 30% of cases of NSCLC (12,17).

The huge family of *bcl-2* genes regulates the next, executive step of the apoptosis signal transduction pathway. Some members of this family like *bcl-2, bcl-X_L, bcl-W, bcl-1, mcl-1, A1 (bfl-1)* or *boo* share both high sequence homology and antiapoptotic properties. In contrast, genes such as *bax, bak, bad, mtd (bok), diva, bik, bid, bim, bcl-rambo, hrk(dp5), blk, bnip3* and *bnip3l*

act as an apoptosis promoters, and their products are characterized by the presence of only one to three Bcl-2 homologous (BH) regions (19). One of the unique features of the Bcl-2 family proteins is heterodimerization between antiapoptotic and proapoptotic proteins, which is considered to inhibit the biological activity of their partners. The increased expression of *bcl-2* gene is observed in proliferating cells. It is also typical of neoplasms, especially those derived from the hematopoietic system (e.g. B lymphocytes in patients with chronic lymphocytic leukemia and non-follicular lymphoma) (15). *Bcl-2* and *bcl-X*$_L$ overexpression resulting in apoptosis inhibition is also common in patients with non-small cell lung cancer (3,11,16).

In the present study we investigated the expression of antiapoptotic genes: *bcl-2* and *bcl-X*$_L$, and the tumor suppressor gene *p53* in patients with NSCLC. The mRNA of these genes was assessed on tumor sections by *in situ* hybridization with '*forward*' and '*reverse*' probes, and the presence and cellular localization of their protein products was evaluated employing immunohistochemistry.

MATERIAL AND METHODS

Patients. A total of 16 patients, diagnosed with non-small cell lung cancer and treated at the Oncology Center of Lublin and at the Clinic of Thoracic Surgery of the Medical University of Lublin in 1997, were reviewed. The mean age of patients was 58 yrs. (range, 41 to 72 yrs.). The stage of the disease was established upon anamnesis, physical examination, X-ray examination of the chest, bronchoscopy, USG examination of abdominal cavity, CT examination of the chest and/or abdominal cavity and biochemical investigation. In cases where alterations in the central nervous system were suspected, a neurological and CT examination of the brain was performed.

According to TNM classification, eight cases of IIB stage were recognized, and eight patients were classified as IIIA stage. Upon histological examination, nine cases were confirmed to be large cell carcinoma, and seven cases to be squamous cell carcinoma. There were no cases of adenocarcinoma. Prior to surgery, all patients were treated with chemotherapy according to the following schedule: Cisplatin (30 mg/m^2 sid) plus Vepesid (100 mg/m^2 sid) for three consecutive days with three cycles of therapy administered every 21 days.

Material. Paraffin embedded tumor specimens from patients that underwent surgery were examined. Four-micrometer sections were cut from paraffin blocks and mounted on slides precoated with poly-L- lysine (SIGMA).

In situ hybridization. Deparaffinization of the specimens was performed as described elsewhere (6). After digestion with proteinase K (Boehringer Mannheim) the sections were incubated overnight with the hybridization mixture, including fluorescein labeled antisense probes (described as *reverse-R*) that were complementary to the mRNA of *bcl-2*, *bcl-X*$_L$ and *p53*. The probes were obtained from Genset Oligos, France. The probe sequences were as follows:

Bcl-2RR: 5'- fCCGCATGCTGGGGCCGTACAGTT - 3'
Bcl-XR: 5'- fGTAGAGTGGATGGTCAGTG - 3'
p53aR1: 5'- CTCAGTTTCCATAGGTCTGAf - 3'

Control reactions were performed for each sample, with sense probes targeting that mRNA (described as *forward-F*). The final staining was performed using NBT/BCIP solution (Boehringer Mannheim), according to manufacturer protocol.

Immunohistochemistry. Deparaffinized sections were incubated with serum-free blocking solution (DAKO) and primary antibodies were applied for 60 minutes at 37^{0}C. Rabbit anti-human bcl-X$_L$ antibody (200 µg/mL, Santa Cruz Biotechnology) was used pre-diluted 1:20 in blocking buffer. Mouse anti-human bcl-2 and mouse anti-human p53 were obtained from DAKO, both ready to use. The negative controls, obtained by substitution of the primary antibody with blocking buffer, were also included in this study. Incubation with secondary antibodies and product visualization was performed employing LSAB2 Kit (DAKO), according to manufacturer protocol. Specimens were then counterstained with Mayer's hematoxyline.

Microscopic examination. After the *in situ* hybridization reaction and immunohistochemistry, the slides were examined with Olympus BH2 light microscope, fitted with S Plan Apo 100 immersion objective. A mean percentage of positive cells was determined in approximately 500 tumor cells. Cases with more than 30% of positive tumor cells were defined as overexpression.

RESULTS

We found an increase in both mRNA and protein expression for the *bcl-X_L* gene in almost all tested patients (100% and 87.5%, respectively). In 10 cases (62.5%), an overexpression of *p53* mRNA was discovered, while the p53 protein was detectable only in 25% of the patients. In the tumor cells of two of the 16 patients (12.5%), bcl-2 protein product was present and 50% (8 cases) showed an increased *bcl-2* mRNA expression. The mean expression of the above genes was also estimated (Tab. 1, Fig. 1).

Table 1. The mean expression of *bcl-2, bcl-X_L* and *p53* genes in patients with non-small cell lung cancer (the percentage of positive tumor cells)

	Number of patients	Bcl-2		Bcl-X_L		P53	
		mRNA	protein	mRNA	protein	mRNA	protein
Squamous	7	34.3	2.6	65.7	65.9	42.8	8.6
Large cell	9	30.0	9.9	69.4	71.1	40.0	20.3
IIB	8	34.4	4.4	55.0	57.2	53.1	29.7
IIIA	8	29.4	9.0	80.6	89.1	29.4	0.6
Total	16	31.9	6.7	67.8	73.2	41.2	15.2

A. The histological type of cancer, B. Stage according to TNM

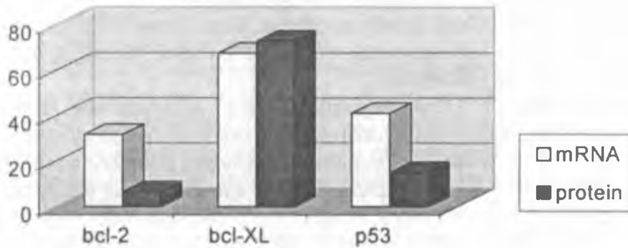

Fig. 1. The mean expression of *bcl-2, bcl-x_L* and *p53* genes in NSCLC patients

The highest mean expression of both mRNA and protein in NSCLC patients was found for the *bcl-X_L* gene. A lower percentage of positive tumor cells was observed in the case of *bcl-2* and *p53* (less than 50%), and the mean expression of the mRNA of these genes was higher than their protein products expression. Microscopic analysis showed that *bcl-X_L* expression was not linked to tumor type. The degree of expression was similar in both squamous cell and large cell carcinomas (Tab. 1, Fig. 2). The mRNA expression of *bcl-2* and *p53* in those histological types was significantly higher (30-42%) than their protein expression (2.57-20.3% of positive cells). Only a slight difference was observed between the *p53* mRNA and protein expression in large cell carcinoma (40% and 20.3%) versus squamous cell carcinoma (42.8% and 8.57%, respectively).

Higher expression of both *bcl*-X_L mRNA and protein was found in IIIA stage carcinoma (according to TNM classification), in comparison with IIB stage (80.6% and 89.1% compared to 55% and 57.2%). In contrast, for *bcl-2* and *p53*, the opposite relation was observed (Tab. 1, Fig. 2).

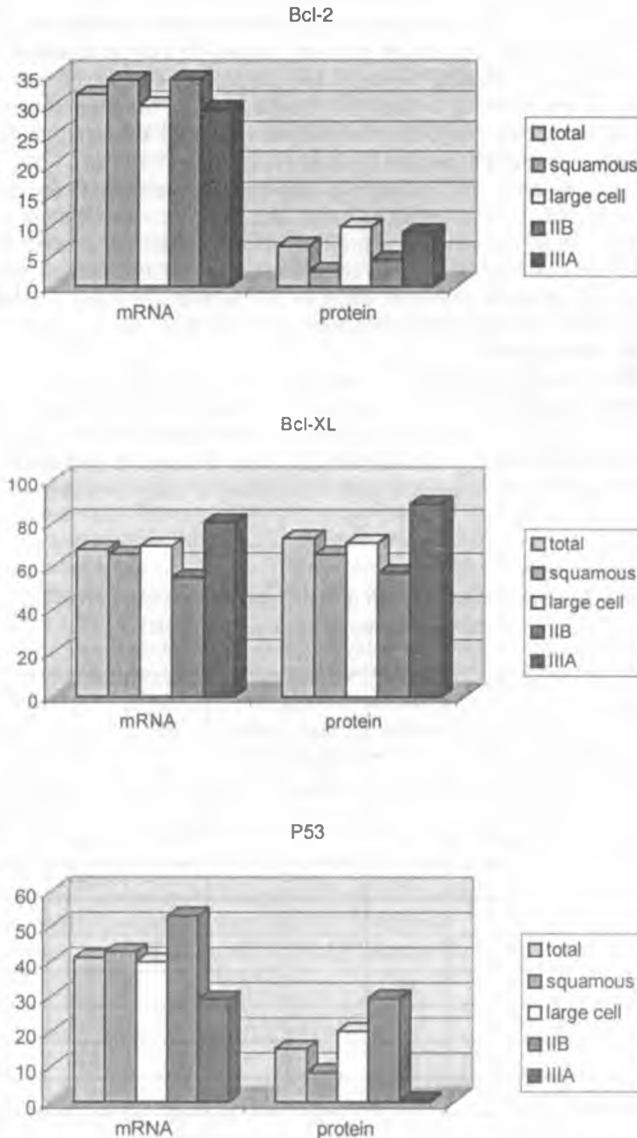

Fig. 2. The expression of *bcl-2, bcl-*x_L and *p53* genes in NSCLC patients depending on histological status and TNM staging

DISCUSSION

The observation of the mechanisms involved in lung cancer pathogenesis constantly brings discoveries of new genes whose mutations or deletions participate in the etiology of this neoplasm. The purpose of studying these gene expressions is to find the ones that may be used as independent diagnostic and prognostic markers as well as those that may be a target of successful therapy.

In cases of non-small cell lung cancer, tumor suppressor genes such as *p53*, *Rb-1*, *cdkn2A* (cyclin-dependent kinase inhibitor 2A) and *fhit* (fragile histidine triad) are extensively investigated (6, 9, 18, 20). The oncogenes *c-myc* and *K-ras* as well as genes belonging to *Bcl-2* family (*bcl-2* and *bcl-X_L*) are also very much of interest (1, 2, 8, 9, 11, 16).

Despite the vast research and widespread publications concerning this issue, the universal prognostic factor for NSCLC has yet to be found, and the data from different research centers is often contradictory. Thus, the aim of our project was to estimate the expression of three chosen genes: *p53*, *bcl-2* and *bcl-X_L* in tumor cells of patients with NSCLC. Our research was performed by investigating gene transcript presence, using *in situ* hybridization on the mRNA level, well as estimating the presence and cellular localization of gene products, using monoclonal or polyclonal antibodies on the protein level.

The gene that most often carries mutations in human neoplasms is the *p53* (18). Physiologically, its expression is low. It increases in response to cellular stress resulting in DNA damage. The wild-type p53 is a short-lived protein, with a half-life ($T^1/_2$) of about 20 minutes, making it almost undetectable by immunochemistry. The presence of p53 in cell smears or tissue sections suggests significantly increased gene expression, or more probably – the gene mutation (18).

Table 2. The mean expression of Bcl-2 and p53 protein in NSCLC cells (the percentage of positive tumor cells)

No.	Author	Number of patients	P53 (%)				Bcl-2 (%)			
			total	PL	MA	AD	total	PL	MA	AD
1	Cynowska	62	48	59	67	28	-	-	-	-
2	Kalogeraki	38	-	-	-	-	-	61.5	-	72
3	Nguyen	89	30	-	-	-	69	-	-	-
4	Ohmura	64	52	-	-	-	44	-	-	-
5	Laudanski	102	54	-	-	-	48	-	-	-
6	Jassem	95	45.2	52.8	0	44.8	-	-	-	-
7	Niklinski	72	40	-	-	-	-	-	-	-
8	Cox	178	43.5	-	-	-	34.9	-	-	-
9	Apolinario	116	64	-	-	-	51	-	-	-
10	Athanassiadou	60	47.7	-	-	-	26.6	-	-	-

PL – squamous cell carcinoma, MA – large cell carcinoma,
AD – adenocarcinoma

Among patients with NSCLC we discovered 4 cases (25%) defined as positive (more than 30% positive tumor cells). According to other authors, p53 protein positive cases in NSCLC range

between 30-54% (Tab. 2). This also depends on the cellular localization of a positive signal – some researchers consider both nuclear and cytoplasmic staining as positive (1), while we took into account only nuclear localization of p53 protein. We observed a higher protein expression in cases of large cell carcinoma in comparison to squamous cell carcinoma (20.3% and 8.6%, respectively), which is generally consistent with the data presented by Cynowska et al. (4). In fact, they describe the percentage of p53 positive tumor cells as 67% for large cell carcinoma and 59% for squamous cell carcinoma. These differences, however, may be attributed to the relatively small group of patients reviewed by us (16 versus 62). In general, the presence of mutated *p53* product is observed more frequently in large cell carcinoma and squamous cell carcinoma as compared to adenocarcinoma (4). The presence of *p53* mRNA transcript, estimated by ISH, was found in 10 patients (62.5%). There is no available data regarding the utilization of this method in the research of *p53* mRNA in NSCLC patients. The increased expression of antiapoptotic genes belonging to the *bcl-2* family (*bcl-2* and *bcl-*X_L) is often described in patients with non-small cell lung cancer (1, 2, 6, 11, 16).

In the course of our research, we employed an anti-bcl-X_L antibody raised against a recombinant protein corresponding to aminoacids 126-188 and mapping at the C-terminus of the Bcl-X_L (not present in Bcl-X_S). We showed the presence of bcl-X_L protein in the cells of 14 patients (87.5%), and by *in situ* hybridization, an increased expression of the *bcl-*X_L gene was found in all tested cases. The percentage of positive tumor cells examined, utilizing both methods, was similar in squamous cell carcinoma and in large cell carcinoma. The increased expression of the *bcl-*X_L gene in NSCLC cells was described previously (11,16), but the data concerned the examination of cell lines derived from this neoplasm.

As to the bcl-2 protein, we found it only in two patients (12.5%), which is less frequent than demonstrated by other authors. The mean protein expression in the presented material was higher in large cell carcinoma cells in comparison to squamous cell carcinoma (9.9% *versus* 2.6%). Kalogeraki et al. (7) investigated 38 NSCLC patients and found bcl-2 protein to be present in 61.5% of cases of squamous cell carcinoma. The mRNA transcript of the *bcl-2* gene, however, was detectable in tumor cells of eight patients (50%). We found no data concerning the *bcl-2* mRNA examination by ISH in NSCLC.

The expression of the investigated genes was also analyzed in the context of the TNM carcinoma staging. However, because of the relatively small group of patients reviewed (IIB – eight patients, IIIA – eight patients), it is difficult to present an objective opinion. We observed significantly lower *bcl-*X_L both mRNA and protein expression in IIB stage as compared to IIIA stage. The *p53* expression was higher in patients classified as IIB stage. Niklinski et al. (14) described the results of *p53* investigation in 72 patients in the I and II TNM stage, and defined its expression as 40%.

The diagnostic methods currently utilized are still not accurate enough to recognize the early stages of this cancer. Our knowledge concerning the alterations of the genes controlling the cell cycle and apoptosis may improve early diagnostics and, in consequence, may help with successful therapy. An unambiguous statement regarding the prognostic value of the investigated genes cannot be made yet, but we believe they will serve as diagnostic markers in the future.

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SUMMARY

Cell neoplastic transformation results from disturbances in expression of genes regulating its basic functions like cell cycle and apoptosis. Our paper presents preliminary estimation of expression of protooncogenes: *bcl-2* and *bcl-X_L* as well as of *p53* suppressor gene in (NSCLC) non-small-cell lung cancer patients. The study comprised 16 NSCLC patients subjected to

chemotherapy before operative procedure. Gene expression was evaluated on paraffin embedded specimens using *in situ* hybridization assay and immunohistochemical method. In the majority of examined patients, high expression of *bcl-*X_L gene both at mRNA and protein level was ascertained. In the case of 10 patients (62.5%), higher *p53* gene expression at mRNA level was observed, whereas higher level of P53 protein was determined only in four subjects (25%). In two of 16 cases (12.5%), protein product of *bcl-2* gene was found, while in eight subjects (50%) – mRNA expression of the gene.

Wstępna ocena ekspresji genów *bcl-2*, *bcl-*X_L i *p53* w lokalnie zaawansowanym niedrobnokomórkowym raku płuca

U podłoża transformacji nowotworowej komórki leżą zaburzenia ekspresji genów, regulujących jej podstawowe funkcje, takie jak cykl komórkowy i śmierć programowana. W naszej pracy przedstawiamy wstępną ocenę ekspresji genów przeciwdziałających apoptozie: *bcl-2* i *bcl-*X_L, oraz genu supresorowego *p53* u chorych z niedrobnokomórkowym rakiem płuc. Badaniem objęto 16 pacjentów z niedrobnokomórkowym rakiem płuca, poddanych chemioterapii przed planowanym zabiegiem operacyjnym. Ekspresję genów oceniano na skrawkach parafinowych metodą hybrydyzacji *in situ* i metodą immunohistochemiczną. U większości badanych stwierdzono wysoką ekspresję genu *bcl-*X_L zarówno na poziomie mRNA, jak i białka. U 10 chorych (62,5%) zaobserwowano podwyższoną ekspresję genu *p53* na poziomie mRNA, zaś podwyższony poziom białka P53 stwierdzono tylko u czterech chorych (25%). W 2/16 przypadków (12,5%) stwierdzono obecność produktu białkowego genu *bcl-2*, natomiast u ośmiu chorych (50%) ekspresję mRNA tego genu.