

APOPTOSIS GENE EXPRESSION PROFILE IN EARLY-STAGE NON SMALL CELL LUNG CANCER

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ABSTRACT

Non small cell lung cancer (NSCLC) is a highly aggressive malignancy with survival rates limited to some patients in early stages (I and II). Apoptosis resistance is a hallmark of solid tumors that is tightly concerned with their biology. We analyzed the expression of 84 apoptosis-related genes in a group of Bulgarian patients with early-stage NSCLC.

RNA samples extracted from 12 early-stage NSCLC patients [five squamous cell carcinomas (SCC) and seven adenocarcinomas (AC)] and eight adjacent non neoplastic pulmonary tissues were used for gene expression analysis. We applied pathway-focused expression profiling of 84 apoptosis-related genes using real-time PCR.

Apoptosis-related genes down regulated in NSCLC compared to non tumor lung tissue ($p < 0.05$) included representatives of the tumor necrosis

factor (TNF) ligand family [TNF superfamily 8 (TNFSF8)], caspase cascade (CASP8 and CASP10) and caspase recruitment domain (CARD) family (BCL10), the positive apoptosis regulator DAPK1 and BCL2 family member MCL1. The potential of apoptosis-related genes as prognostic and predictive markers should be validated in future studies.

Keywords: Apoptosis, Early-stage, Non small cell lung cancer (NSCLC), Expression

INTRODUCTION

Lung cancer is the leading cause of cancer-related death worldwide [1]. It is the most common cancer in men (1.1 million cases, 16.5% of total cases), while in females, lung cancer is the fourth most frequent cancer (513,000 cases, 8.5% of all cancers) [2]. In Bulgaria, the age-standardized incidence rates of lung cancer by sex per 100,000 population are 72 for men and 11.1 for women [1]. According to their histological type, 80-85% of lung cancer cases belong to non small cell lung cancers (NSCLC), of which 35-40% are adenocarcinomas (AC), 25-30% squamous cell carcinomas (SCC) and 10-15% large cell cancers [3]. Worldwide AC is more frequent than SCC in women (55% vs. 25%), compared to men (30% vs. 57%) [4], while in Bulgaria SCC is more frequent in both sexes: 25.9 vs. 17.0% for women and 41.9 vs. 6.3% for men [5].

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Survival rates in NSCLC cases depend on the tumor stage at diagnosis, the 5-year survival rate of patients with resected NSCLC being between 50-60% [6,7]. The current tumor node metastasis (TNM) system remains inaccurate for prediction of individual patients' survival, as 50% of patients with early-stage NSCLC will develop recurrent disease [8]. Thus, it is important to identify patients with the highest likelihood for recurrence who may potentially benefit the most from adjuvant chemotherapy. Assessment of a patient's prognosis could be improved by combining standard clinical variables such as tumor stage and histology with intrinsic genetic characteristics of the tumors.

Apoptosis is an evolution-conserved and genetically regulated form of programmed cell death, which plays an important role in maintenance of tissue homeostasis. Resistance to apoptosis is a fundamental property of human cancers [9]. General resistance of NSCLC to a diversity of cytotoxic agents suggests a deregulation of apoptotic signaling [10]. Of clinical importance is the identification of novel prognostic markers for recurrence and patients' stratification in early stages of the disease. We have analyzed the expression of 84 apoptosis regulators in a group of Bulgarian patients with early-stage NSCLC.

MATERIALS AND METHODS

The study includes 12 patients with primary NSCLC who were admitted to the Clinic of Thoracic Surgery, St. Sofia University Hospital, Sofia, Bulgaria, between November 2007 and July 2008 and underwent a lobectomy for resection of tumor nodules. None had received prior therapy. Tissue acquisition was approved by the Institutional Ethics Committee and all participants signed informed consent forms. We analyzed 12 tumor samples and eight adjacent non cancerous lung tissues (as controls). All tumors were staged post-operatively according to the classification system of the International Union Against Cancer (UICC) and were early-stage NSCLCs. Five patients had squamous cell carcinoma (SCC) and seven patients had adenocarcinoma (AC). Clinical characteristics of the patients are summarized in Table 1.

Total RNA was extracted from the tissue samples (RNeasy MiniKit; Qiagen, Hilden, Germany) and genomic DNA contamination was eliminated (RNase-free DNase set; Qiagen). RNA concentration was measured spectrophotometrically and the RNA integrity of all samples was tested by denaturing agarose electrophoresis. One µg of each RNA sample was used for cDNA synthesis (High Capacity Reverse Transcription Kit; Applied Biosystems, Foster City, CA, USA).

Table 1. Clinical data about the analyzed lung cancer cases

Tumor ID	Sex-Age	Histological Subtype	Stage	Grade	Lymph Node Metastasis
T1	M-52	AC	IB	G3	[-]
T2	M-59	AC	IB	G2-G3	[-]
T3	M-73	AC	IA	G2	[-]
T4	M-49	AC	IA	G1	[-]
T5	F-52	AC	IB	G2	[-]
T6	M-63	AC	IB	G3	[-]
T7	M-54	AC	IA	G2	[-]
T8	F-59	SCC	IA	G1	[-]
T9	M-69	SCC	IA	G1	[-]
T10	M-54	SCC	IA	G2-G1	[-]
T11	M-68	SCC	IIB	G3	[+]
T12	F-73	SCC	IIB	G2	[+]

Polymerase chain reaction (PCR) was performed on a 7500 Real Time PCR System (Applied Biosystems). Gene expression was analyzed using Human Apoptotic RT² Profiler PCR Array (SuperArray Bioscience Corporation, Qiagen, Hilden, Germany).

For expression analyses we used web-based software (available at www.sabiosciences.com), which automatically performs fold-change calculations based on the $\Delta\Delta C_t$ method. The house-keeping gene RPL13A was used for data normalization. For each tumor, the fold change of each gene was calculated compared to the mean value of its expression in the control group. A gene was considered to be down or up regulated if its expression was altered more than 4-fold in at least half of the analyzed NSCLCs.

For statistical evaluation of the data we used the Student's *t*-test and the non parametrical tests of Shapiro-Wilk and Mann-Whitney. A *p*-value of 0.05 was accepted as the threshold for significant difference between the tumor and non tumor samples (Table 2).

RESULTS

Using the web-based PCR Array Data Analysis software we calculated the expression of each gene

in the tumor tissue compared to the mean value of expression in adjacent non tumor tissues. Genes with altered expression and their average fold regulation values are presented in Table 2.

Using the set criterion, none of the 84 analyzed genes were up regulated, while six genes were down regulated in the tumor tissues. Of the underexpressed genes, two were caspases and members of the death effector domain family, CASP8 and CASP10. Other substantially down regulated genes include BCL10 containing caspase recruitment domains (representing the CARD family) and tumor necrosis factor superfamily 8 (TNFSF8), a TNF ligand family member. The death domain-containing gene DAPK1 showed the most stable underexpression being down regulated in eight of the 12 NSCLC cases. From the BCL2 related family members, slight down regulation was shown for MCL1 (Table 2).

We performed separate analyses of gene expression in the AC and SCC histological subtypes of NSCLC. In AC, we found the BCL2 family member HRK to be down regulated in five out of seven cases, while in SCC it was up regulated in four out of five cases although it was below the set threshold in three of them. The SCC histotype showed underexpression of DAPK1 in four out of five cases, while four out of seven ACs showed underexpression of this gene.

Table 2. Genes down regulated in tumor vs. control samples

Gene	Description	Samples With Down Regulation	Fold Change	Fold Regulation	p Value
BCL10	B-cell CLL/lymphoma 10	6/12	0.06 ± 0.02	-37.34	0.047
CASP10	Caspase 10, apoptosis-related cysteine peptidase	6/12	0.09 ± 0.04	-28.50	0.013
CASP8	Caspase 8, apoptosis-related cysteine peptidase	6/12	0.09 ± 0.02	-13.18	0.011
DAPK1	Death-associated protein kinase 1	8/12	0.13 ± 0.02	-9.11	0.003
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	6/12	0.18 ± 0.01	-5.49	<0.001
TNFSF8	Tumor necrosis factor (ligand) superfamily, member 8	6/12	0.10 ± 0.03	-16.47	0.008
HRK	Harakiri, BCL2 interacting protein (contains only BH3 domain)	5/7 (AC)	0.14 ± 0.02	-8.57	0.854

2 Fold change and fold regulation values are expressed as means of the tumors which showed downregulation of the respective genes. Fold change values are presented as means ± standard error of the mean (SEM). AC - adenocarcinoma

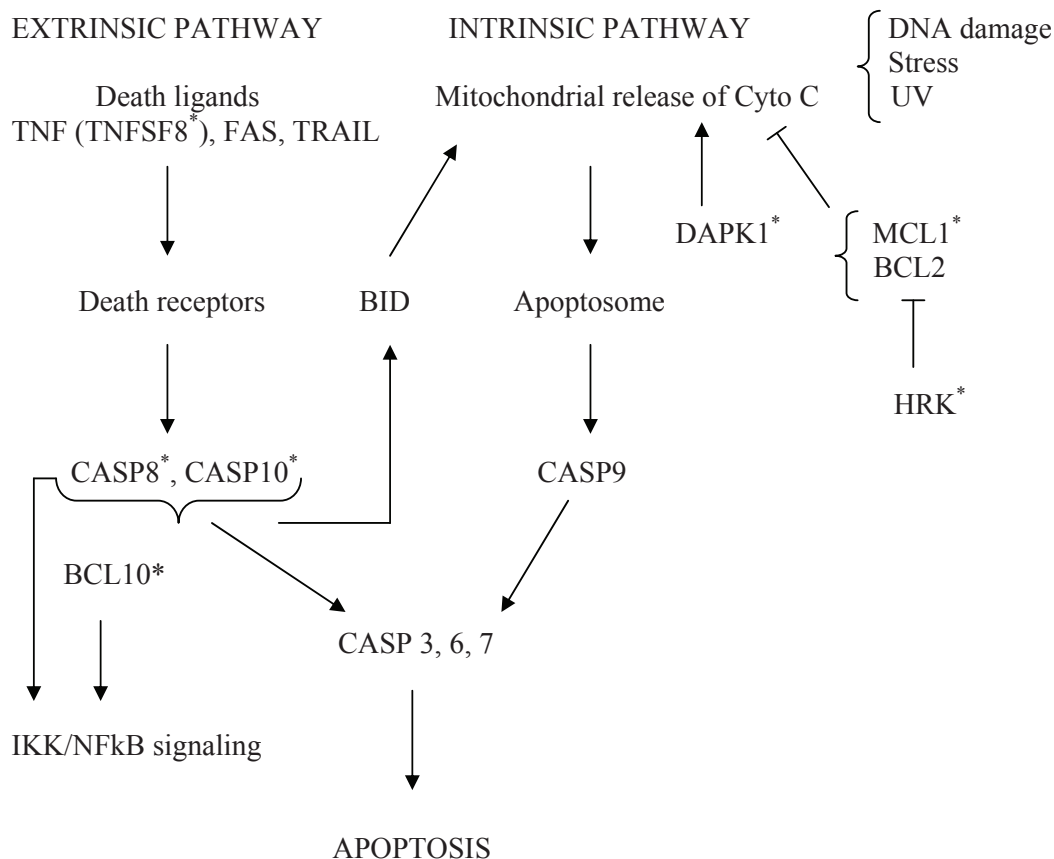


Figure 1. Schematic presentation of the down regulated genes (*) and their place in the apoptotic pathways.

DISCUSSION

Apoptosis occurs via an extrinsic and an intrinsic pathway. The extrinsic pathway is initiated by binding of cell surface death receptors (via death domains) to adaptor proteins [e.g., Fas-associated via death domain (FADD)] in a death-induced signaling complex. The intrinsic pathway acts through generation of mitochondrial permeability transition leading to the establishment of the ‘apoptosome’ protein complex. Both pathways converge into a common cascade that consists of proteolytic enzymes-caspases [11]. The down regulated genes and their role regarding the two apoptotic pathways are presented in Figure 1.

The extrinsic pathway is initiated via activation of the cell surface death receptors by members of the TNF superfamily. Expression analysis of our NSCLC samples suggests deregulation of TNF-mediated cell death via down regulation of the TNF ligand TNFSF8 and of DAPK1, calcium-calmodulin regulated protein kinase [12] involved

in mitochondrial-based pro-apoptotic events [13]. While the role of TNFSF8 in lung tumorigenesis is not clear, the down regulation of DAPK1 is consistent with previous findings [14] and is probably caused by aberrant methylation [15-17].

Among the substantially down regulated genes were CASP8 and CASP10, initiator caspases which transmit apoptotic signals [18]. Their death-effector domains interact with that of the adaptor FADD, which is involved in TNF-related apoptosis [19]. Loss of CASP8 through gene hypermethylation [20] allows for cellular survival in the stromal microenvironment, and promotes metastases [21]. BCL10, another proapoptotic gene, which along with CASP8 is involved in activation of NFkB signaling [22], was the most significantly down regulated gene in our NSCLC samples.

The intrinsic pathway is composed of various pro- and anti-apoptotic members of the BCL2 family. In NSCLC, we found slight but constant down regulation of the BCL2 family member MCL1, which may induce apoptosis by overexpression of

its short isoform [23]. HRK, another BCL2-related activator of apoptosis, was under expressed in AC histotype but not in the SCC.

Our results suggest that deregulation of apoptosis in early-stage NSCLC is associated with down regulation of factors of TNF-mediated cell death (including TNFSF8, and the kinase DAPK1), caspase cascade (via initiator caspases CASP8 and CASP10), and the CARD domain containing mediator BCL10. A larger group of patients is needed in order to confirm the above results, while their clinical significance as potential prognostic and predictive markers should be validated in future studies.

REFERENCES

1. Ferlay J, Parkin DM, Steliarova-Foucher E. Estimates of cancer incidence and mortality in Europe in 2008. *Eur J Cancer*. 2010; 46(4): 765-781.
2. Lung cancer incidence and mortality worldwide in 2008. Summary. (<http://globocan.iarc.fr/factsheets/cancers/lung.asp>).
3. Lonardo F, Li X, Kaplun A, Soubani A, Sethi S, Gadgeel S, Sheng S. The natural tumor suppressor protein maspin and potential application in non small cell lung cancer. *Curr Pharm Des*. 2010; 16(16): 1877-1881.
4. Sculier JP, Chansky K, Crowley JJ, Van Meerbeeck J, Goldstraw P; International Staging Committee and Participating Institutions. The impact of additional prognostic factors on survival and their relationship with the anatomical extent of disease expressed by the 6th Edition of the TNM Classification of Malignant Tumors and the proposals for the 7th Edition. *J Thorac Oncol*. 2008; 3(5): 457-466.
5. Valerianova ZG, Vukov MI, Dimitrova NE, Eds. *Cancer Incidence in Bulgaria 2006*, 1st ed. Sofia: AVIS-24 Ltd, 2008.
6. Zochbauer-Muller S, Gazdar AF, Minna JD. Molecular pathogenesis of lung cancer. *Annu Rev Physiol*. 2002; 64: 681-708.
7. Pignon JP, Tribodet H, Scagliotti GV, Douillard JY, Shepherd FA, Stephens RJ, Dunant A, Torri V, Rosell R, Seymour L, Spiro SG, Rolland E, Fossati R, Aubert D, Ding K, Waller D, Le Chevalier T; LACE Collaborative Group. Lung adjuvant cisplatin evaluation: a pooled analysis by the LACE Collaborative Group. *J Clin Oncol*. 2008; 26(21): 3552-3559.
8. Goldstraw P, Crowley J, Chansky K, Giroux DJ, Groome PA, Rami-Porta R, Postmus PE, Rusch V, Sobin L; International Association for the Study of Lung Cancer International Staging Committee; Participating Institutions. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM classification of malignant tumours. *J Thorac Oncol*. 2007; 2(8): 706-714.
9. Fulda S. Tumor resistance to apoptosis. *Int J Cancer*. 2009; 124(3): 511-515.
10. Horn L, Sandler A. Epidermal growth factor receptor inhibitors and antiangiogenic agents for the treatment of non-small cell lung cancer. *Clin Cancer Res*. 2009; 15(16): 5040-5048.
11. Schulz WA. Apoptosis and replicative senescence in cancer. In: Schulz WA, Ed. *Molecular Biology of Human Cancers*. Dordrecht: Springer Science + Business Media. 2005: 145.
12. Kang BN, Ahmad AS, Saleem S, Patterson RL, Hester L, Doré S, Snyder SH. Death-associated protein kinase-mediated cell death modulated by interaction with DANGER. *J Neurosci*. 2010; 30(1): 93-98.
13. Jang CW, Chen CH, Chen CC, Chen J, Su YH, Chen RH. TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase. *Nature Cell Biol*. 2001; 4(1): 51-58.
14. Mashkova TD, Oparina NI, Zinov'eva OL, Kropotova ES, Dubovaia VI, Poltarau AB, Fridman MV, Kopantsev EP, Vinogradova TV, Zinov'eva MV, Laktionov KK, Kasymova OT, Zborovskaia IB, Sverdlov ED, Kiselev LL. Transcription TIMP3, DAPK1 and AKR1B10 genes in squamous cell lung cancer. *Mol Biol (Mosk)*. 2006; 40(6): 1047-1054.
15. Han W, Wang T, Reilly AA, Keller SM, Spivack SD. Gene promoter methylation assayed in exhaled breath, with differences in smokers and lung cancer patients. *Respir Res*. 2009; 10: 86.
16. Wang Y, Zhang D, Zheng W, Luo J, Bai Y, Lu Z. Multiple gene methylation of nonsmall cell lung cancers evaluated with 3-dimensional microarray. *Cancer*. 2008; 112(6): 1325-1336.
17. Liu Y, Gao W, Siegfried JM, Weissfeld JL, Luketich JD, Keohavong P. Promoter methylation of RASSF1A and DAPK and mutations of K-ras,

p53, and EGFR in lung tumors from smokers and never-smokers. *BMC Cancer*. 2007; 7: 74.

18. Logue SE, Martin SJ. Caspase activation cascades in apoptosis. *Biochem Soc Trans*. 2008; 36(Pt. 1): 1-9.

19. Neumann L, Pforr C, Beaudouin J, Pappa A, Fricker N, Krammer PH, Lavrik IN, Eils R. Dynamics within the CD95 death-inducing signaling complex decide life and death of cells. *Mol Syst Biol*. 2010; 6: 352.

20. Yang Q, Kiernan CM, Tian Y, Salwen HR, Chlenski A, Brumback BA, London WB, Cohn SL. Methylation of CASP8, DCR2, and HIN-1 in neuroblastoma is associated with poor outcome. *Clin Cancer Res*. 2007; 13(11): 3191-3197.

21. Stupack DG, Teitz T, Potter MD, Mikolon D, Houghton PJ, Kidd VJ, Lahti JM, Cheresch DA. Potentiation of neuroblastoma metastasis by loss of caspase-8. *Nature*. 2006; 439(7072): 95-99.

22. Su H, Bidere N, Zheng L, Cubre A, Sakai K, Dale J, Salmena L, Hakem R, Straus S, Lenardo M. Requirement for caspase-8 in NF-kappa-B activation by antigen receptor. *Science*. 2005; 307(5714): 1465-1468.

23. Bae J, Leo CP, Hsu SY, Hsueh AJW. MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain. *J Biol Chem*. 2000; 275(33): 25255-25261.