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Expression of pro-apoptotic signalling molecules mRNA in lung cancer

Ekspresja mRNA w raku płuca molekuł sygnałowych aktywujących apoptozę

Lung cancer is the leading cause of cancer mortality worldwide. Despite using carboplatin oxaliplatin, paclitaxel, docetaxel, gemcitabine, vinorelbine and camptothecin derivates, limited advances in the treatment for lung cancer were achieved. One of the main problems in cancer therapy is resistance to apoptotic stimuli. Most drugs and radiotherapy cause apoptosis via DNA damage or inhibition of enzymes that play a pivotal role in cell survival. In both pathways, initiation of apoptosis is brought about by stimulation of pro-apoptotic proteins such as Bad, Bid, Bax and Back which cause channels formation in external mitochondria membrane. The consequence of these changes is releasing of some molecules like cytochrom *c*, Smac/DIABLO (Smac, second mitochondria-derived activator of caspases/direct IAP binding protein with low pI), AIF (Apoptosis Inducing Factor) or Omi/HtrA2 (Omi) from mitochondrial intermembrane space [9]. Cytochrome c was the first recognised as a mitochondrial-released activator of apoptosis via caspase pathway. Mechanism of apoptosis promotion is different for cytochrome *c* and Smac. Smac inhibits so-called inhibition of apoptosis proteins (IAPs), i.e. cIAP-1, cIAP-2, XIAP and survival, which inactivate caspases 3 and 7 and thus allows to progress of apoptosis. In cancer cells Smac is released not only via direct mitochondrial pathway but also by extracellular death receptor signalling. It is very likely that activation of caspase 8 by receptor signalling is not strong enough to stimulate the next control points on apoptosis pathway. Therefore, mitochondrial amplification of signal coming from the receptor is required to inhibit proteases responsible for inactivation of pro-apoptotic caspase 3, 6 and 7 by Smac. Smac is known to potentiate apoptosis by counteracting the anti-apoptotic function of the IAP's. All IAP's containing at least one BIR (baculovirus IAP repeat) domains. It was found that e.g. XIAP inhibits the activity of caspases via BIR domain. When mitochondrial Smac is released into cytosol it interacts with the BIR2 and BIR3 domains of XIAP and thus protects inhibition of caspase 9 activity. Caspase 9 has a similar tetrapeptide motif in its NH_2-terminus, so both compete for the BIR3 domain of XIAP. Capase-3 is released by the interaction between NH_2-terminus of Smac and BIR2 domain of XIAP [3]. As a result, cascade activation by catalytic cleavage is begun, thereby inducing apoptosis.

Omi is regulated by environmental factors such as heat shock or chemical stress. Similarly as Smac, the protein is located in mitochondrial intermembrane space. In both instances of the mitochondrial or receptor apoptotic pathway, Omi is released to cytoplasm and it causes increase of caspase 9 activity. Furthermore, Omi can promote apoptosis in a caspase-independent pathway through its ability to function as a specific protease. Unlike Smac, any interaction between Omi and survivine has not been observed. Moreover, Omi shows stronger tissue expression than Smac. Several studies have shown that Smac overexpression or Smac-mimetic compound stimulates neoplastic cells to apoptotic death [5]. This molecule might induce apoptosis in cancer cells exhibiting high expression of XIAP and c-IAP1 or amplifying pro-apoptotic signals in cells with low IAPs expression stimulated by TRAIL (tumor necrosis factor (TNF)-related apoptosis-inducing ligand) or etoposide. Smac can be used to sensitize cells that are deficient in pro-aopoptotic Bax and Bak genes, or resistant to TRAIL. Smac-mimetic compounds may activate the NF-κB pathway, depending on c-IAP1 and c-IAP2 degradation [13], which sensitizes TNF-dependent apoptosis via caspase 8 [11]. Consistent results were obtained from animals' research. Smac peptides amplify apoptotic signals induced by TRAIL in glioma cells and stimulated tumor regression [2]. Hepatocellular carcinoma tumor regression was also observed when nude mice with xenografted tumors were locally treated with 5-fluorouracil and an adenovirus expressing Smac [16]. Thus, in that light the recognition of the control points that could regulate cell division and apoptotic process might be very useful for the development of new anticancer strategies and as a predictive marker. The aim of the study was to investigate level of pro-apoptotic signals coming from pre-mitochondrial and mitochondrial sources in lung cancer depending on selected clinico-morphological parameters.

MATERIAL AND METHODS

The study included 39 males with the average age of diagnosis of 61.5 years (range: 46 to 81years). On diagnosis, patients were treated with excision of tumor. In 20 cases it was pulmonectomy, in 3 cases – bilobectomy, in 10 cases – lobectomy and in 6 cases – tumorectomy. The hstological type of tumor and grading was based on World Health. After histological examination, total RNA was isolated from paraffin embedded tissue using RNeasy FFPE–kit (Qiagen, Germany). Paraffin tissue slides (10 μm) were treated with xylene and incubated in lysis buffer containing proteinaze K for 15 minutes. The samples were kept for 15 minutes at 80˚C to reverse RNA modifications caused by formalin. Then, DNA presented in the samples was removed using column system. RNA was condensed, purified, reversely transcribed and amplified using one step method from Qiagen. The following primer sequences were used:

- for p53 – CCCAGCCAAAGAAGAAACC and reverse primer GAACAAGAAGT-GGAGAATGTCAGT;
- for smac /Diablo – GAAGCATTGATGAGGAGAGCAG and reverse primer GCTCT-GGCTCCTATGATCACC;
- for Omi/HtrA2 – GGGGAGCAGATGGTACAAAA and reverse primer CAGAAC-CTCAGCCAGAAAGG.

To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification with oligonucleotide primers for β-actine: GATCATTGCTCCTCCTGAGC and CACCTTCACCGTTCCAGTTT. Using Eppendorff mastercykler (Niemcy) gradient, 255-bp, 212-bp, 194-bp, and 308-bp products were obtained for p53, Smac/Diablo, Omi/HtrA2 and β-actine, respectively. Cycling conditions were as follows: 50˚C for 30 min. reverse transcription; initial denaturation at 95˚C for 15 min; followed by 30 cycles of 94˚C for 45 sec. denaturation, and

56˚C (β-actine, Smac and Omi) or 53˚ (p53) for 30 sec. annealing, and 72˚C for 1 min extension. Final extension was conducted at 72˚C for 10 min. Amplified products were separated on a 2% agarose gel, and bands were visualized by ethidium bromide and photographed under ultraviolet transillumination to confirm the specificity of the bands. The intensity of the bands was estimated using 1D Image Analysis Software (Kodak). In every tested tissue, all measured parameters were calculated in relation to β-actine. The statistical analysis was performed using STATISTICA 5. Statistical significance was defined at a p value ≤ 0.05.

RESULTS

One can observe that p53, Smac and Omi mRNA relative expressions were lower in G2 than in G3 stage in samples coming from squamous cell carcinoma (Table 1), but no statistical significance was seen. Unlike squamous cell carcinoma, adenocarcinoma samples showed higher p53 and Omi mRNA relative expression in G2 than G3 (Table 2). In these cases there were also no differences between the tested groups. A comparison of adenocarcionoma versus squamous cell carcinoma (Table 3) showed that there were no significant differences in relative expressions of mRNA for every tested parameter. With regard to age (Table 4), one can observe lower values in the younger versus the older group for all tested molecules, but there were no significant differences.

Table. 1. p53, Smac and Omi mRNA relative expressions in squamous cell carcinoma depending on histological stage

Molecul	Grading	N	Min	Max	M	SD	p
p53	G2	18	43.00	139.00	75.06	28.85	0.133
	G3	10	49.00	145.00	93.40	29.97	
Smac	G2	18	45.00	138.00	84.67	28.69	0.053
	G3	10	71.00	140.00	107.60	23.85	
Omi	G2	13	38.00	131.00	80.38	37.37	0.323
	G3	6	56.00	216.00	103.00	59.42	

Table 2. p53, Smac and Omi mRNA relative expressions in adenocarcinoma depending on histological stage

Molecul	Grading	N	Min	Max	M	SD	p
p53	G2	7	62.00	121.00	82.86	20.80	0.186
	G3	4	26.00	137.00	6675	48.52	
Smac	G2	7	52.00	104.00	92.00	18.12	0.449
	G3	4	59.00	135.00	93.50	31.29	
Omi	G2	4	52.00	150.00	98.00	44.68	0.564
	G3	4	54.00	90.00	73.25	19.45	

Table 3. p53, Smac and Omi mRNA relative expressions depending on type of cancer (Adeno. – adenocarcinoma; Squamous – squamous cell carcinoma)

Molecul	Histologic type	N	Min	Max	M	SD	p
p53	Adeno.	11	26.00	137.00	77.00	32.12	0.674
	Squamous	28	43.00	145.00	81.61	30.06	
Smac	Adeno.	11	52.00	135.00	92.55	22.16	0.974
	Squamous	28	45.00	140.00	92.86	28.87	
Omi	Adeno.	8	52.00	150.00	85.63	34.54	0.916
	Squamous	19	38.00	216.00	87.53	45.04	

Table 4. p53, Smac/DIABLO and Omi mRNA relative expressions in lung cancers depending on age

Molecules	Age	N	Min	Max	M	SD	p
p53	>65	14	43.00	137.00	85.79	29.27	0.409
	≤65	24	26.00	145.00	77.13	31.68	
Smac	>65	14	52.00	135.00	94.36	27.20	0.639
	≤65	24	45.00	140.00	90.13	26.29	
Omi	>65	10	42.00	150.00	96.00	36.73	0.396
	≤65	17	38.00	216.00	81.65	44.33	

DISCUSSION

It has been reported that apoptosis-associated genes are down- or up-regulated in human carcinoma cells. The p53 protein plays a major role in apoptosis via two mechanisms. Firstly, p53 acts as a transactivator of proapoptotic genes like Bax, Noxa, and PUMA. Bax induces apoptosis by enhancing the release of mitochondrial proteins, e.g., cytochrome *c* and Smac to cytosol [12]. The second mechanism depends on repression of the down-regulated antiapoptotic genes (e.g. Bcl-2) that promote apoptosis. The p53 protein translocates to mitochondria in response to cellular stress, resulting in apoptosis via interaction with antiapoptotic Bcl-2 and Bcl-XL proteins, thus altering the mitochondrial membrane potential and inducing cytochrome *c* and Smac release into the cytosol causing caspases activation [14]. As a result, apoptotic death events are observed. That phenomenon could not be seen in case of mutation in p53 gene revealed in many types of malignances [4]. Moreover, overexpression of Bcl-2 and Bcl-X_L may contribute to resistance to chemotherapy [8].

High Smac mRNA expression was observed in testis and at lower levels in the heart, liver, kidney and prostate. The lowest expression was seen in the brain, lung and leucocytes of the blood [10]. The expression of Smac was also described in e.g. colorectal, lung, and ovarian carcinomas [15]. However, many human cancers do not express Smac mRNA. In those cases resistance to apoptosis could be expected, thereby promoting their survival. In our studies the expression of pro-apoptotic molecules at the pre-mitochondrial and mitochondrial level – p53, Smac and Omi mRNAs were compared in two distinct histological types of lung carcinomas: adenocarcinoma and squamous cell carcinoma. The level of all tested mRNA was comparable in all tested samples, thus it seems that sensitivity of cells to chemotherapeutic stimuli regulated by p53, Smac and Omi might be similar in both types of tumors. These observations are consistent with the results obtained by Sekimura et al. [7], who revealed the expression of Smac mRNA in primary lung cancer. The comparison of Smac mRNA expression in cancer and normal lung specimens showed that Smac mRNA level was lower in these types of tumours than in normal tissue. The prognosis of patients with a tumour exhibiting low expression of Smac mRNA was worse than in those with a normal

lung exhibiting high mRNA expression. The authors postulated that Smac expression may play a role in the carcinogenesis, progression and prognosis of primary lung cancer. However, in another research, significantly higher expression of Smac mRNA in non-small cell lung carcinoma (NSCLC) was found as compared to the normal tissue [6]. High expression was also observed in ten different NSCLC cell lines. Smac transcript was simultaneously upregulated in a subset at NSCLCs various histopathological types, grade and stage categories. In our studies we observed lack of differences in p53 Smac and Omi mRNA expression according to the histopathological type, grade and age categories. A similar discrepancy was seen in two studies with renal cell carcinoma (RCC). One demonstrated that Smac expression at both mRNA and protein level was not associated with the stage and grade of tumor. However, another study showed a significant inverse correlation between Smac protein expression level and both the stage and histological grade of RCC. The differences might probably result from the number of samples and stability of used housekeeping genes. De Boever and co-workers [1] proved differences in stability of most frequently applied housekeeping gens i.e., beta-actin, glyceraldehyde-3-phosphate dehydrogenase, hypoxanthine phosphoribosyl-transferase, ubiquitin and glucose-6-phosphate dehydrogenase using real time PCR.

CONCLUSIONS

In tested specimens, the level of apoptotic signalling represented by p53, Smac/DIABLO and Omi/Htr2 is comparable independently of age, adenocarcinoma/squamous cell carcinoma type and grade of tumor differentiation.

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SUMMARY

The main features of cancer cells that distinguish them from normal cells are uncontrolled synthesis of molecules that stimulate growth and insensitivity to apoptotic stimuli such as radiation or chemotherapy. Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP binding protein with low pI) is a pro-apoptogenic mitochondrial protein that is released from mitochondria similarly to cytochrom *c,* AIF, Omi/HtrA2. Smac/DIABLO inhibits proteases and in that way caspases may avoid inactivation, which allows progress of apoptosis pathway. Better understanding of a signal apoptotic transduction in cancer cells gives hope to obtain drugs that could be used in cancer therapy. The aim of the study was to investigate the level of proapoptotic signals coming from pre-mitochondrial and mitochondrial sources in lung cancer depending on selected clinico-pathological parameters. The p53, Smac/DIABLO and Omi/Htr2 mRNA relative expression was evaluated in lung cancer specimens taken from 39 male patients. The study revealed lack of significant differences in p53, Smac/DIALO and Omi/Htr2 mRNA expression according to the histological type and grade of cancer differentiation as well as age categories. It could be concluded that the level of apoptotic signalling represented by p53, Smac/DIABLO and Omi/Htr2 in the tested specimens is comparable despite the tested parameters.

STRESZCZENIE

Jedną z cech komórek raka jest niezależność od sygnałów ustrojowych, co m.in. prowadzi do niekontrolowanych podziałów oraz oporności na sygnały proapoptotyczne, indukowane przez promieniowanie jonizujące i chemoterapeutyki. Smac/DIABLO jest proteiną o działaniu proapoptotycznym, uwalnianą z mitochondriów, podobnie jak cytochrom *c,* AIF, Omi/HtrA2. W cytoplazmie hamuje proteazy odpowiedzialne za degradację kaspaz. Lepsze poznanie mechanizmów przekazywania sygnałów promujących apoptozę w komórkach raka daje nadzieję na otrzymanie nowych, bardziej skutecznych leków przeciwnowotworowych. Celem badań była ocena ekspresji molekuł proapoptotycznych na poziomie przedmitochondrialnym i mitochondrialnym w raku płuca w zależności od wieku, typu histologicznego i stopnia zróżnicowania nowotworu. Oceniano względną ekspresję mRNA dla p53, Smac/DIALO i Omi/Htr2. Badania wykazały brak znamiennych różnic w ekspresji mRNA dla badanych molekuł sygnałowych między grupami otrzymanymi według przyjętych kryteriów. Wyniki badań sugerują, że natężenie sygnałów proapoptotycznych pochodzących od p53, Smac/DIALO i Omi/Htr2 jest podobne dla raka gruczołowego i płaskonabłonkowego, porównywanych grup wiekowych oraz stopnia złośliwości histologicznej.