Survivin antiapoptotic gene expression as a prognostic factor in non-small cell lung cancer: *in situ* hybridization study

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Abstract: Survivin is an inhibitor of apoptosis that plays a significant role in cell cycle regulation and is important for survival prognosis in many neoplasms. Survivin expression was assessed by in situ hybridization (ISH) in 60 consecutive patients (54 males and 4 females) with NSCLC treated between 1993 and 1997. The examined patients had IIB and IIIA stage according to TNM system. In all cases the chemotherapy with cisplatin and etoposide (2 cycles) was administered prior the surgery; in patients responding to the therapy one more cycle was applied. Survivin gene overexpression was observed in 35 patients (58.3%). There was no correlation between survivin mRNA level and histological type of tumor, stage of cell differentiation, stage of disease according to TNM classification, performance status according to WHO and number of chemotherapy regimens administered ($p > 0.05$). However, the correlation between survivin gene expression and response to the chemotherapy was statistically significant ($p = 0.04$). Statistical analysis showed that median survival in patients with survivin gene overexpression was shorter (14.0 months) as compared to patients with no expression (60.0 months; $p = 0.00002$). In survival assessment by means of Kaplan-Meier test, 14.3% of five-year survival was achieved in the former group versus 60% in the latter ($p = 0.00003$). Univariate analysis (log-rank test) showed that significant independent prognostic factors in NSCLC included: stage of the disease according to TNM classification ($p = 0.006$), response to chemotherapy ($p = 0.005$) and pattern of survivin gene expression ($p = 0.00003$). Multivariate analysis utilizing Cox's model showed that for survival assessment the stage according to TNM, response to the chemotherapy and survivin expression estimated by means of ISH are of statistical significance ($p=0.00001$). The calculated predictive values showed that ISH technique was quite accurate in assessment of five-year survival. Our data show that survivin expression may be used as a prognostic factor and a target for therapy.

Key words: Survivin - *In situ* hybridization - Non-small cell lung cancer

Introduction

Apoptosis is a specific form of cell death that follows degradation of intracellular polypeptides by proteases known as caspases. There are two main pathways of caspase activation. First one is activated by the ligands of death receptors, the second one by various stress factors like DNA damage or microtubule structure alterations. Activation of this proteolysis cascades involves various polypeptides like Bcl-2 family proteins, protein kinases and IAP (Inhibitor of Apoptosis) family pro-

teins. One of IAP proteins, survivin, has been cloned in 1997 by Ambrozini and coworkers [3]. The gene for survivin has been mapped to chromosome 17q25; it encodes a protein of 16.5 kDa [3, 20, 21].

Survivin is the only described apoptosis inhibitor that is expressed in cell cycle-dependent manner. Its expression increases in G_2/M phase and decreases rapidly in G_1. In addition to regulation of apoptosis, it has been found to be involved in the regulation of mitosis by interacting with mitotic spindle apparatus. Survivin overexpression initiates cell division by inducing resistance to G_1 arrest and thereby accelerating the entry into S phase [11]. Survivin as apoptosis inhibitor requires special mention because of lack of its expression in normal adult tissues and significant overexpression in tumor cells [3, 4, 10,

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11]. Survivin overexpression was demonstrated in many neoplasms like breast cancer, prostate, gastric, colon, bladder, esophageal carcinomas, lymphomas, neuroblastomas, osteosarcomas and lung cancer [1-3, 5, 7-10, 12-14, 17-18, 22-24].

The aim of our study was survivin expression analysis in NSCLC with regard to clinical and histopathological characteristics, five-year survival assessment depending on clinical and histopathological characteristics and analysis of prognostic value of survivin gene expression in survival time estimation.

Materials and methods

Patients. A total of 60 consecutive patients with non-small cell lung cancer treated at the Oncology Center of Lublin Land and at the Clinic of Thoracic Surgery of the Medical University in Lublin between 1993 and 1997, were reviewed. There were 6 women and 54 men in the group examined; mean age of patients was 57 yrs (range 38 to 69 yrs). The stage of the disease was established upon anamnesis, physical examination, X-ray examination of the chest, bronchoscopy (followed by cytological and/or histopathological examination), USG examination of abdominal cavity, CT examination of the chest and/or abdominal cavity and biochemical investigations.

According to TNM classification, 26 cases were recognized as IIB stage (43.3%) and 26 patients had IIIA stage (56.7%). The staging was revised according to TNM classification from 1997 [19]. Upon histological examination, 46 cases were confirmed to be squamous cell carcinoma (76.7%), 9 cases to be adenocarcinoma (15%) and 5 cases to be large cell carcinoma (8.3%).

Also, the performance status (PS) according to WHO was estimated. PS was 0 in ten patients (16.7%) and 1 in 60 patients (83.3%). All patients were treated with chemotherapy according to the following schedule: Cisplatin (30 mg/m^2) plus Vepesid (100 mg/m^2) for 3 days; administered every 21 days.

After 2 cycles, the efficiency of the therapy was checked by physical examination, X-ray examination of the chest, bronchoscopy, CT examination of the chest and/or abdominal cavity and biochemical investigations. In case of disease progression (cPD) or stabilization (cNC) patients were immediately qualified to surgery, while cases with partial (cPR) or complete regression (cCR) were treated with one more cycle of chemotherapy and than qualified to surgery. One patient with cPR did not agree for chemotherapy continuation, he was qualified for surgery. In the group examined all patients underwent surgery.

Specimen preparation. Formalin-fixed, paraffin-embedded tumor specimens from patients that underwent surgery were examined. Four-micrometer sections were mounted on slides precoated with poly-L-lysine (Sigma). The presence of survivin gene transcript was assessed by means of *in situ* hybridization.

***In situ* hybridization.** Deparaffinization of the specimens was performed utilizing xylene, as described elsewhere [16]. First, the specimens were warmed up to 56˚C for 10 min. Then they were washed with xylene (3 × 5 min), absolute ethanol (2 × 3 min), 90% ethanol (3 min) and finally with distilled water [16]. After digestion with proteinase K (Boehringer Mannheim, 20 μg/ml in 10 mM TRIS-HCl, pH 7.5; 15 min at 37˚C) the sections were washed twice with PBS and incubated overnight at 37˚C in humidified chamber with the hybridization buffer (Sigma) including fluorescein-labeled antisense probe (described as reverse-R). This probe that was complementary to the mRNA of survivin was obtained from Genset Oligos, France. The probe sequence was as follows: 5'-fAGC GCA ACC GGA CGA ATG CTT -3'.

Control reactions were performed for each sample, with sense probe targeting that mRNA (described as forward-F). The additional control reaction involved incubation with hybridization buffer lacking the probe. Incubation conditions followed manufacturer's instructions. After extensive washing with PBS (3 × 5 min with agitation) blocking buffer was applied (Dako) for one hour at 37˚C, followed by the incubation with sheep anti-fluorescein antibody alkaline phosphatase-conjugated (Roche), 1:200 in blocking buffer (2 h at 37˚C). The specimens were than washed with PBS (3 × 5 min) and the final staining was performed using NBT/BCIP solution (Boehringer Mannheim) for 12 h, according to manufacturer protocol. Endogenous alkaline phosphatase activity was blocked with 0.1 μM levamisole (Sigma). The specimens were then counterstained with hematoxylin and eosin.

Microscopic examination. After the *in situ* hybridization reaction, the slides were examined with Olympus BH2 light microscope, fitted with S Plan Apo 100 immersion objective. We looked for the presence of RNA transcript visualized as navy-blue granules [15].

Statistical analysis. The qualitative variables were analyzed by two-way tables. Significance of differences between two qualitative variables was tested using the chi-square Pearson test. The Yeates correction was applied for two-by-two tables and small expected frequencies. The survival time was assessed starting on the first day of chemotherapy cycle. The 5-year survival was evaluated by means of survival tables and total survival-by the Kaplan-Meier test. The survival times were compared with the log-rank test and Cox proportional hazard model was used. 5% inference error was accepted and statistical significance was ascribed to p < 0.05. Median follow-up was 22 months (range 5-60 months).

To establish exactness of our tests, the sensitivity and specificity of the method was assessed as well as its positive and negative predictive value in the context of five-year survival and tests accuracy, according to the following formulas, where: PU = true negative results, PD = true positive results, FU = false negative results, FD - false positive results.

Sensitivity (%) = PD/PD+FU = probability of positive test result in patients that died.

Specificity (%) = PU/PU+FD = probability of negative test result in patients that survived over 5 years.

PPV (positive predictive value) = PD/PD+FD = probability of death based on positive test result.

NPV (negative predictive value) = PU/PU+FU = probability of survival based on negative test result.

ACC accuracy = (PU+PD): number of patients = probability of positive test result in patients that died and negative test result in patients that live.

The analysis was performed using Statistica v. 6.0 software.

Results

Specimens from consecutive 60 NSCLC patients were analyzed. Survivin gene transcript was present in 35 patients (58.3%). An overexpression of survivin gene was observed in all 6 women, while in men the presence of survivin transcript was found in 53.7% of cases (29 patients) (p = 0.08). In the examined group with positive survivin expression, there were 62% patients with performance status (PS) 1 (p = 0.35). While analyzing survivin mRNA presence with regard to clinical stage we found more cases at IIIA according to TNM classification, although it was not statistically significant (p = 0.54). In the group of 25 patients with no survivin expression, 80% (20 patients) showed response to the

Table 1. Clinicopathological characteristics and survivin gene expression in patients with non-small cell lung cancer

Characteristics		In situ hybridization				P value
		Survivin-positive		Survivin-negative		
		No.	%	No.	%	
Number of patients (%)		35	58.3	25	41.7	
Sex	Females	6	100,0	0	0,0	0.08
	Males	29	53.7	25	46.3	
Performace status	PS-0	4	40,0	6	60.0	0.35
	PS-1	31	62,0	19	38.0	
Staging according to TNM	II B	14	53.9	12	46.1	0.54
	III A	21	61.8	13	38.2	
Histology	Adenocarcinoma	5	55.6	4	44.4	0.14
	Large cell carcinoma	5	100,0	0	0,0	
	Squamous cell carcinoma	25	54.4	21	45.6	
Stage of cell differentiation	G2	15	51.7	14	48.3	0.32
	G3	20	64.5	11	35.5	
Number of chemotherapy cycles	2	16	72.7	6	27.2	0.09
	3	19	50.0	19	50.0	
Response to chemotherapy	cCR + cPR*	19	54.3	20	80.0	0.04
	cNC + cPD*	16	45.7	5	20.0	

*cCR - clinical complete response, cPR - clinical partial response, cND - clinical stable disease, cPD - clinical progression disease

chemotherapy (cCR and cPR). This result was statistically significant (p = 0.04).

In the material examined, no significant correlation was observed between the pattern of survivin expression assessed by ISH and histological type of tumor, stage of cell differentiation and number of chemotherapy regimens administered (Table 1).

In the examined group we observed 33.3% patients that survived 5 years. The highest number of deaths due to local renewal took place during first two years (32 patients - 80%). In 8 patients renewal and/or distant metastases were diagnosed during five-year follow-up.

Among 20 patients that survived 5 years, 12 were classified as IIB stage according to TNM and 8 were classified as IIIA stage. All these patients showed response to chemotherapy (cPR), one of them showed complete response (cCR), he had IIIA stage of disease.

Clinicopathological characteristics were included into univariate Cox regression model. There were statistically significant differences in five-year assessment with regard to TNM stage (p = 0.006), response to the chemotherapy (p = 0.005), survivin gene expression (p = 0.00003) (Tables 2, 3).

Patients with increased survivin gene expression showed significantly shorter median survival in comparison to patients with no survivin expression (14.0 months vs. 60.0 months), p = 0.00002. Kaplan-Meier survival curves of NSCLC patients are demonstrated in Figure 1.

Five-year survival in the group of patients with survivin overexpression was 14.3%, while in patients with no expression it was 60% (p = 0.00003).

Table 4 presents significant predictors in multivariate Cox regression model, involving stage of disease according to TNM classification, response to the chemotherapy and survivin gene expression. For survival assessment, the stage according to TNM, response to the chemotherapy and survivin expression estimated by means of ISH are of statistical significance (p=0.00001).

The calculated predictive values show that ISH method is quite accurate in five-year survival estimation (Table 5).

Discussion

Survivin, a recently identified IAP family member, is a bifunctional protein that causes inhibition of apoptosis and regulates cell cycle [1, 21]. Survivin transcript or protein expression was observed in many neoplasms like: breast cancer (70.7%), prostate cancer (82%), gastric cancer (34.5%), colon cancer (53.2%), esophageal cancer (63; 70,7%; 83.3%), lymphomas (60%), neuro-

Table 2. Univariate analysis of 5-year survival with regard to clinicopathological characteristics

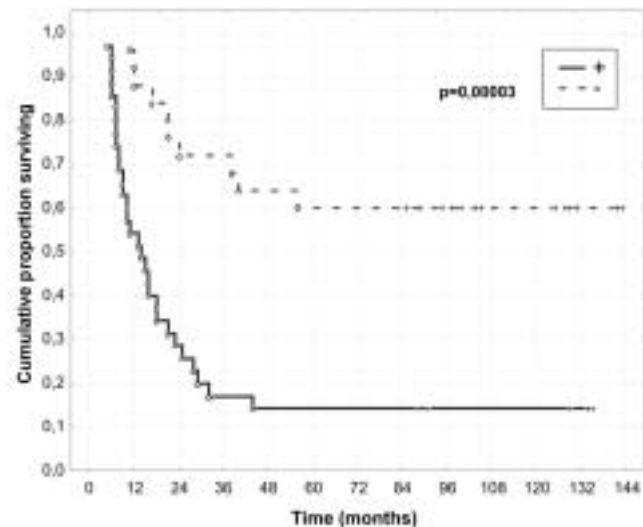| Characteristics		Number of patients	5-year survival (%)	P value
Sex	females	6	16.7	0.1
	males	54	35.2	
Performance status (PS) acc. to WHO	0	10	50	0.13
	1	50	30	
Staging according to TNM	IIB	26	46.2	0.006
	IIIA	34	23.5	
Histology	Squamous cell ca	46	39.1	0.13
	Adenocarcinoma	9	11.1	
	Large cell ca	5	20.0	
Stage of cell differentiation	G2	29	34.5	0.65
	G3	31	32.4	
Response to chemotherapy	cCR+cPR	39	43.6	0.005
	cNC+cPD	21	14.3	

Table 3. Univariate analysis of five-year survival with regard to survivin gene expression

Survivin gene expression	Positive	Negative	P value
Median survival time (months)	14.0	60.0	0.00002
Five-year survival (%)	14.3	60.0	0.00003

Fig. 1. Kaplan-Meier curves for overall survival rates of patients with NSCLC categorized according to survivin expression (ISH).

blastomas (47%) and osteosarcomas (57.5%) [1-3, 5, 9, 10, 12-14, 17, 23, 24]. Survivin overexpression was found in lung cancer, but it was not observed in other lung tissue lesions [22].

The recent literature presents a lot of data about survivin gene expression in cancer estimated by RT-PCR, while little is known about survivin transcript presence estimation directly within tissue by *in situ* hybridization (ISH). There is only one study by Ambrosini and coworkers [3] showing ISH of survivin in squamous lung carcinoma, colon adenocarcinoma and breast carcinoma.

We employed *in situ* hybridization to assess survivin gene expression in non-small cell lung cancer. In the examined group of 60 patients we found survivin mRNA expression in 58.3% of cases at IIB and IIIA stage according to TNM classification. Some authors examined survivin expression in NSCLC by RT-PCR; they showed its presence in 36.8-96% of cases [6, 7, 18, 22]. Falleni *et al.* [7] analyzed the expression of survivin

mRNA in non-small cell lung cancer at IA and IB stages, they found it in 96% of cases and observed increased expression in squamous cell carcinoma (p = 0.0022) [7]. Monzo and coworkers [18] examined 83 patients; they found survivin expression in cancer cells of 71 patients (85.5%). They also showed survivin mRNA in non-cancer cells in 12% of cases. At IIIA stage of disease according to TNM classification, survivin overexpression was observed in 27/32 patients. There was no correlation between survivin expression and age, sex, histological type of tumor, stage of differentiation, tumor size or lymph node involvement [18].

We did not find survivin transcript in normal tissues. In the material examined, no significant correlation was observed between the pattern of survivin expression assessed at mRNA level and histological type of tumor, stage of cell differentiation, stage according to TNM classification, performance status according to WHO and number of chemotherapy regimens administered. However, the correlation between survivin gene expression and response to the therapy was statistically significant (p = 0.04)

Survivin overexpression is associated with poor prognosis, as also proved in our study. In material examined by ISH we found that high expression of survivin was correlated with short survival (median survival time was 14 months) (p = 0.00002). Monzo *et al.* [18] reported that in 12 patients showing the absence of survivin mRNA, significantly longer median survival was observed as compared to 71 patients that were survivin-positive (p = 0.01). Median survival time in the group of patients with positive survivin expression was 19 months.

In patients with esophageal cancer that showed partial response to chemotherapy (cPR), survivin expres-

Table 4. Multivariate model of Cox regression

Characteristics	Estimation of parameters	SE	Hazard ratio	P value
TNM	-1.22	0.35	0.3	0.00004
Response to chemotherapy	0.26	0.11	1.3	0.02
Survivin expression	1.45	0.39	4.27	0.0002

Table 5. Prognostic values of survivin mRNA assessment by ISH for survival estimation

Parameter	ISH (%)
Sensitivity	75,0
Specificity	75,0
Positive predictive value	85,7
Negative predictive value	60,0
Accuracy	75,0

sion estimated by RT-PCR was significantly lower than in patients with stable disease (cNC) and progressive disease (cPD) [12]. In the available literature, no data was found about the effect of survivin transcript presence on response to chemotherapy in NSCLC. In patients that showed no survivin expression we found response to the chemotherapy (clinical complete response - cCR or clinical partial response - cPR) in 80% of cases. It included patients with clinical partial response and one patient who showed complete clinical response (p = 0.04). The pattern of survivin expression may thus reflect susceptibility to the chemotherapy.

The calculated predictive values showed that ISH method was quite accurate in five-year survival estimation. Survivin gene overexpression is definitely a poor prognostic factor.

In conclusion, our results demonstrate that survivin in NSCLC may be identified as an independent prognostic factor, and as apoptosis inhibitor may stand for the therapeutic target in cancer therapy.

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