Expression of Survivin in Squamous Cell Lung Cancer and Its Correlation with Prognosis

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ABSTRACT

There are outstanding studies on the importance of survivin and inhibition of apoptosis in many cancers, including lung. Evaluation of the relationship between survivin expression in lung cancer and prognosis is the aim of this study. A total of 50 patients with a diagnosis of squamous cell lung carcinoma were included in this study. Survivin levels that were obtained with IHC (immunohistochemical) and RT-PCR (reverse transcription-polymerase chain reaction) methods from the tumor and metastatic lymph node tissues embedded in paraffin blocks were evaluated along with clinical parameters. The median age of the patients was 60 years (range 54-69). All patients were male and all of them were smokers. The mean duration of follow-up and disease-free survival was 42.08 ± 22.46 months and 40.62 ± 22.46 months, respectively. Significant associations were found among survivin levels in metastatic lymph nodes and duration of smoking (IHC and RT-PCR), angiolymphatic invasion (IHC), the number of mitosis (RT-PCR) ($p= 0.05$ and $p= 0.05$, $p< 0.001$, $p= 0.02$, respectively). Survivin showed statistically significant associations with angiolymphatic invasion and stage in multivariate analysis ($p< 0.001$, $p= 0.04$, respectively). An association was not found between survivin levels in tumor and lymph nodes and survival. Higher survivin levels in lymph nodes detected by IHC was associated with shorter survival but that didn't reach statistical significance (65.22 ± 5.16 vs 42.33 ± 11.97, $p= 0.72$). Further larger studies done with larger numbers of patients are required in order to evaluate its effects in our society.

Keywords: Survivin, Squamous cell lung cancer, Survival

ÖZET

Skuamoz Hücreli Akciğer Kanserinde Survivin Ekspresyonu ve Prognostik Korelasyonu

Akciğer kanseri de dahil olmak üzere birçok kanserde apopitozis inhibisyonu ve survivinin önemine değinen önemli çalışmalar bulunmaktadır. Bu çalışmanın amacı akciğer kanserinde survivin ekspresyonu ile prognoz ilişkisini değerlendirmektir. Skuamoz hücreli akciğer kanseri tanılı toplam 50 hasta çalışmaya alındı. Tümör ve metastatik lenf nodlarında survivin düzeyi İHK (İmmunhistokimya) ve RT-PCR (reverz transkripsiyon-polimeraz zincir reaksiyonu) yöntemleri ve klinik parametrelerle beraber değerlendirildi. Hastaların median yaş değeri 60 yıl (sınırlar 54-69) idi. Hastaların hepsi erkekti ve hepsi sigara içicisiydiler. Ortalama takip süresi 42.08 ± 22.46 ay, hastalıksız sağkalım süresi ise 40.62 ± 22.46 ay olarak hesaplandı. Metastatik lenf nodundaki survivin düzeyiyle, sigara içme süresi (İHK ve RT-PCR), anjiyolenfatik invazyon varlığı (İHK) ve mitoz sayısı (RT-PCR) arasında anlamlı ilişki saptandı (sırasıyla $p= 0.05$, $p= 0.05$, $p< 0.001$, $p= 0.02$). Mutivariet analizde survivin ile anjiyolenfatik invazyon varlığı ve evre arasında anlamlı ilişki bulundu (sırasıyla, $p< 0.001$, $p= 0.04$). Tümör ve lenf nodundaki survivin seviyesi ile sağkalım arasında anlamlı ilişki bulunamadı. Lenf nodunda İHK ile saptanan yüksek survivin düzeylerinde sağkalım daha kısa olmakla beraber istatistiksel olarak anlamlılık saptanmadı. Toplumumuzda survivinin prognostik ve prediktif önemini belirlemek için daha fazla hasta sayısıyla yapılacak daha kapsamlı çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Survivin, Skuamoz hücreli akciğer kanseri, Sağkalım

INTRODUCTION

Lung cancer consists 12% of all cases of cancer globally. It occupies the second place among causes of cancer-related deaths in males and females.[1,2] Smoking is the most important etiologic factor in the development of lung cancer. The risk of lung cancer is 20 times higher in smokers in comparison to non-smokers. Age to start smoking, total duration of smoking, total number of cigarettes smoked, are all effective on the risk.[3]

Recently, some targets related to adenocarcinoma subtype were detected and drugs targeting them were developed, but for squamous cell carcinoma until now there is no such type of specific target. The first line of therapy for this histological type is chemotherapy. Studies on determination of biomarkers for predicting prognosis and response to chemotherapy have increased recently.

Apoptosis is a process which is important in both carcinogenesis and cancer treatment.[4-6] There are outstanding studies on the importance of survivin and inhibition of apoptosis in many cancers, including breast, lung, esophagus, stomach, head-neck, and cervix.[7-11]

Survivin is a protein which is made up of 142 amino acids of 16.5 kD, and is an apoptosis inhibitor. It is the smallest member of IAP (inhibitor apoptotic protein) family in mammals.[12] According to some studies surviving may act as a target for cancer treatment and as a prognostic marker.[13-14]

There is no data on the literature evaluating the correlation between survivin in squamous cell subtype and clinicopathological parameters and survival. There are recent notable studies evaluating the relationship between especially response to chemotherapy, EGFR resistance and surviving.[15-18]

In this study, the relationship between survivin in both tumor and metastatic lymph nodes and clinicopathological parameters and survival in patients with squamous cell non-small cell lung cancer, which is more frequently seen in our country were evaluated. The study was approved by the Ethics Committee of Pamukkale University and scientific investigation project.
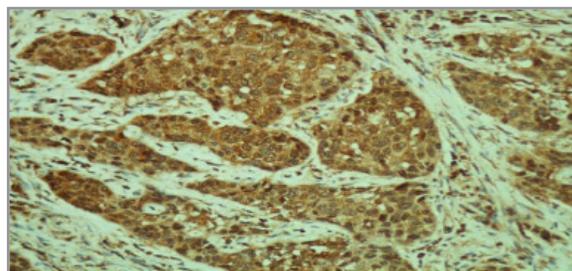
PATIENTS AND METHODS

A total of 50 patients with squamous cell lung car-cinoma, stages between 1-3, who had undergone pneumonectomy, lobectomy, or wedge resection, who had not received neoadjuvant chemotherapy or radiotherapy, treated and followed-up by the Pamukkale University Medical School Department of Medical Oncology between the years 2005-2013 were examined retrospectively. The demographic data of the patients, tumor types, chemotherapies received were extracted from the patient files. The patients were detected from the patient follow-up files of Outpatient Clinics of Medical Oncology, and the stages, duration of survival were examined statistically at the end of study.

Determination of survivin expression with IHC method: The diagnosis of lung carcinoma, tumor differentiation in the histological sections of resection materials, and the presence of metastatic lymph node in lymph node dissection materials were re-examined and re-evaluated. The samples that best reflected the tumor tissue were selected. Five 3 micron sections were taken for the purpose of survivin antibody examination to positive – charged glasses from selected paraffin blocks for each case. The tissue samples were left at 50^0C for one night in the incubator for deparaffination. They were later dyed automatically with the help of VENTANA, Benchmark LT device. In sections dyed automatically, 1/200 diluted survivin antibody (Anti-BIRC5 policlonal rabbit antibody, BiOSs, Woburn, USA) was used and the targeted proteins were visualized. The duration of incubation with antibodies was 36 minutes for survivin. After which, it was held in 70% alcohol for 2 minutes, in 80% alcohol for 2 minutes, in 95 % alcohol for 2 minutes and absolute alcohol for 2 minutes, sequentially. The sections were dried in the air and held in Xylene for 15 minutes, and closed with entellan.

Survivin expression was determined in the cytoplasm. Cytoplasmic staining was considered positive for survivin, and other staining were considered negative in the evaluation. Cytoplasmic survivin staining was recorded in between 0-100 % according to diffusiveness. Staining of 50% and more of the cells was considered as an increased survivin expression (Picture 1).

Determination of survivin expression with RT-PCR method: Both tumor tissues of patients and also 10 μm sections of lymph nodes in terms of distant or-

Picture 1. Higher expression level of survivin in poorly differentiated tumors

Table 1. The histopathological characteristics of the patient

Characteristics	Patient, n (%)	Mean±SD
Age (year)	-	61.94±7.61
Gender		
Male	50 (100)	
Female	0 (0)	
Smoking		
Yes	50(100)	
No	0 (0)	
Smoking time (month)	-	29.60±8.91
Operation		
Lobectomy	29 (58)	
Pneumonectomy	20 (40)	
Wedge resection	1 (2)	
Lymph node metastasis		
Yes	21 (42)	
No	29 (58)	
Stage		
1-2	45(90)	
3	5 (10)	
Tumor localization		
Central	33 (66)	
Peripheral	17 (34)	
Tumor differentiation		
Poor	12 (24)	
Moderate-well	38 (76)	
Tumor mitotic count	-	7.86±3.05
Angiolymphatic invasion		
Yes	28 (56)	
No	22 (44)	
Adjuvant chemotherapy		
Yes	22 (44)	
No	28 (56)	
Adjuvan radiotherapy		
Yes	3 (6)	
No	47 (94)	
Metastasis		
Yes	9 (18)	
No	41 (82)	
Progression-free survival (month)	-	40.62±22.46
Overall survival (month)	-	42.08±20.68

gan metastasis were transported to sterile ependorf tubes. These were incubated at 56° C for 20 minutes with xylene in order to remove paraffin from these tissues. Afterwards, paraffin was removed by centrifugation at 14000xg for 2 minutes. After this procedure, xylene was removed with use of 96% absolute alcohol. After centrifugation at 14000 xg for 2 minutes, the supernatant was removed. The pellet was incubated at 56°C for 15 minutes for evaporation of alcohol from the environment. Then, RNA was isolated with use of qiagen FFPE isolation (kat:73504 Germany) kit. The RNA thus obtained was measured with a nanodrop and was stored at -80°C. After total RNA levels of all patients were obtained, total RNAs were turned into cDNA with the help of qiagen cDNA (Kat:679923 France) synthesis kit. The protocol was as follows; 5x tampon 5μl, dNTP 100 nM, random nanomer 5,2 μl, reverse transcriptase 1 μl, DTT 1,25 μl, RNAse inhibitor $0,5\mu$l, sample 10 μl reaction complex were prepared. cDNA was obtained after incubations at 65^0C for 5 minutes, on ice for 5 minutes, at 25^0C for 10 minutes, at 50^0C for 60 minutes and at 85^0C for 5 minutes and measured at nanodrop. For investigation of real-time PCR with obtained cDNA, survivin (Roche Applied Science Basel Switzerland), caspase 9 (Roche Applied Science Basel Switzerland) and beta actin (Roche Applied Science Basel Switzerland) gene expressions were examined. The reaction conditions were as follows: 10 minutes at 95^0C for 1 cycle, at 95^0C for 10 seconds, at 60^0C for 30 seconds, at 72^0C for 1 second (reading) for 45 cycles and cooling was done at 40^0C for 30 seconds. Calculations were done with the ratio of obtained beta actin cycle number with the other target gene (delta ct). Median values were determined from results obtained

from tumor and lymph nodes. Survivin values of 1.4 and more in the tumor, and 0.52 and over in the lymph node were considered as increased.

Statistical Analysis

SPSS version 17 was used in the statistical analysis. Descriptive statistics were presented as mean, standard deviation and percent. Significance of differences between the groups was evaluated

Table 2. Associations between survivin levels in metastatic lymph nodes and histopathological characteristics of the patients

Level of survivin in metastatic lympnode						
			IHC		RT-PCR	
Characteristics		n	Mean±SD	p	Mean±SD	p
Age (year)	<65	32	19.44±32.80	0.09	0.33±0.82	0.70
	>65	18	37.03±42.67		0.44±0.96	
Smoking time	≤30 pocket/year	23	20.65±36.19	0.05*	0.12±0.43	0.05*
	>30 pocket/year	27	39.25±41.68		0.58±1.07	
Mitotic count	Low (≤7)	26	30.57±39.65	0.88	0.27±0.97	0.02*
	High (>7)	24	30.83±41.16		0.48±0.74	
Operation	Lobectomy	29	22.06±37.35	0.09	0.38±0.98	0.73
	Pneumonectomy	20	44.75±41.15		0.37±0.71	
	Wedge resection	1	1.00±0.00		1.00±0.00	
Stage	2	45	26.33±39.57	0.03*	0.36±0.89	0.28
	3	5	70.00±14.14		0.50±0.68	
Tumor localization	Peripheral	17	29.24±40.11	0.63	0.36±0.97	0.32
	Central	33	33.52±40.76		0.39±0.65	
Tumor differentiation	Poor	12	14.16±33.15	0.09	0.31±0.56	0.72
	Moderate-well	38	35.92±40.89		0.39±0.95	
Angiolymphatic invasion	Yes	28	50.17±41.91	<0.001*	0.43±0.88	0.36
	No	22	5.90±17.90		0.30±0.86	
Metastasis	Yes	9	33.53±41.20	0.47	0.41±0.91	0.65
	No	41	17.77±32.70		0.20±0.62	

* Univariate analysis is obtained and analysed by using Mann Whitney U and Kruskal-Walles tests. p< 0.05 is accepted to be statistically significant

with Mann-Whitney U test. Associations between quantitative data were determined with the help of Spearman correlation test. Progression-free and overall survival was calculated with Kaplan–Meier. Log-rank was used for statistical significance and p< 0.05 was considered as statistically significant. In evaluation of tumor and lymph node survivin levels in multivariate analysis, linear regression was used and cox regression analysis was used for evaluation of survival duration.

RESULTS

The median age of 50 patients included in this study was 60 years (range, 54-69). All of the patients were males and all had a history of past smoking. Twenty-two (44%) patients had received adjuvant chemotherapy, 3 patients (6%) had adjuvant radiotherapy. Twenty nine patients had undergone (58%) lobectomy, 20 had undergone pneumonectomy (40%), 1 patient (2%) had undergone wedge resection. In histopathological examination, 12 of the patients had poor differentiated morphology, whereas 38 showed moderate-well differentiation. The tumor diameter was 1 cm at its smallest point, and 10 cm at its largest, with a mean value of 4.01±2.09 cm. The tumor had a central location in 33 (66%) patients, and peripheral location in 17 (34%) patients. In histopathological examination, the mean number of mitosis in the tumor was 7 (range, 4-10). Of the patients, 28 (56.0%) had angiolymphatic invasion, and 21 (42%) had lymph node metastasis. Forty-five patients (90%) were at stage 1 and 2, 5 patients (10%) were at stage 3. The mean duration of follow-up was 42.08±20.68 months in 50 patients. The mean disease-free duration of survival of the patients was 40.62±22.46 months, and the general duration of survival was 42.08±20.68 months. The histopathological characteristics of the patients are presented in Table 1. The mean survivin level in the tumor was 74.70±27.30 with IHC method; and

Table 3. Associations between survivin levels in the tumor and lymph node and OS

	Mean±SD	p
Age (year)		
≤65	62.23±4.27	0.78
>65	63.58±5.46	
Lymph node metastasis		
No	63.66±5.10	0.79
Yes	61.05±4.65	
Stage		
1-2	62.79±3.62	0.97
3	60.00±10.73	
Angiolymphatic invasion		
No	64.33±4.33	0.59
Yes	59.43±5.67	
Tumor differentiation		
Poor	62.15±4.01	0.73
Moderate-well	62.25±6.29	
Adjuvant Chemotherapy		
No	50.45±4.78	0.01
Yes	72.52±2.39	
Metastasis		
No	73.46±1.51	p<0.001
Yes	18.33±2.36	
Smoking time		
<30 pocket year	66.98±3.34	0.02
≥30 pocket year	47.26±8.10	
Level of survivin in tumor cell by IHC		
Low (<%50)	60.97±3.49	0.73
High (>%50)	59.62±9.41	
Level of survivin in tumor cell by RT-PCR		
Low (≤1.39)	62.04±4.52	0,62
High (>1.39)	61.26±4.90	
Level of survivin in metastatic lympnode by IHC		
Low (<%50)	65.22±5.16	0.72
High (>%50)	42.33±11.97	
Level of survivin in metastatic lympnode by RT-PCR		
Low (≤0.51)	68.50±5.21	0.32
High (>0.51)	61.07±4.06	

* Univariate analysis is obtained and analysed by using Mann Whitney U and Kruskal-Walles tests.

30.70±39.9 in the lymph node. According to extensiveness of cytoplasmic survivin staining, values between 0-100% were found. Staining of 50% and more of the cells was considered as evidence of increased survivin expression. As there are no studies in the medical literature examining survivin levels in metastatic lymph nodes, survivin in lymph nodes was considered as in the tumor. The mean survivin value in the tumor was found to be 2.44±2.94 with PCR method, and 0.37±0.86 in the lymph node. For the survivin values determined with PCR method, threshold value for the tumor was 1.39 with ROC analysis and it was 0.51 for the lymph node. No correlations were found between survivin levels determined with IHC and PCR methods in the tumor. Also, there were no associations between survivin levels determined with IHC and PCR methods in the lymph nodes (p> 0.05). As it is shown in Table 2, significant associations were found between survivin levels in metastatic lymph nodes and the duration of smoking (IHC and RT-PCR), angiolymphatic invasion (IHC) and the number of mitosis (RT-PCR) (p= 0.05 and p= 0.05, p< 0.001, p= 0.02, respectively). There was a significant association between angiolymphatic invasion and stage with survivin levels in multivariate analysis (p< 0.001, p= 0.04, respectively). In survivin levels which were found to be increased in the lymph node with IHC, OS was shorter but it did not reach statistical significance (65.22±5.16 vs 42.33±11.97, p= 0.72). An association was not found between survivin levels in the tumor and lymph node and OS (Figure 1 and 2). Only lymph node metastasis were significantly correlated with OS in multivariate analysis (p= 0.001) (Tables 3, 4).

Table 4. Associations between survivin levels in the tumor and lymph node and OS in multivariate analysis

	Beta	95%CI		p value
Adjuvant chemotherapy	6.03	0.45	80.93	0.17
Metastasis	112.78	7.35	1725.53	0.001*
Smoking time	0.83	0.14	4.78	0.84
Level of survivin in tumor cells by IHC	1.04	0.10	10.38	0.96
Level of survivin in metastatic lympnode by IHC	3.04	0.16	55.60	0.45

* p values are obtained by lineer regression analysis. p < 0.05 is accepted to be statistically significant
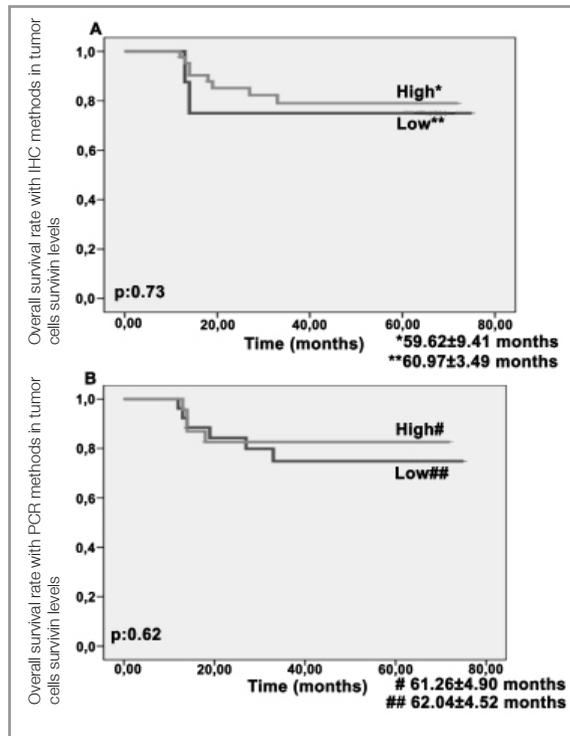
Figure 1. (A) Overall survival rate with IHC methods in tumor cells survivin levels; **(B)** Overall survival rate with PCR methods in tumor cells survivin levels
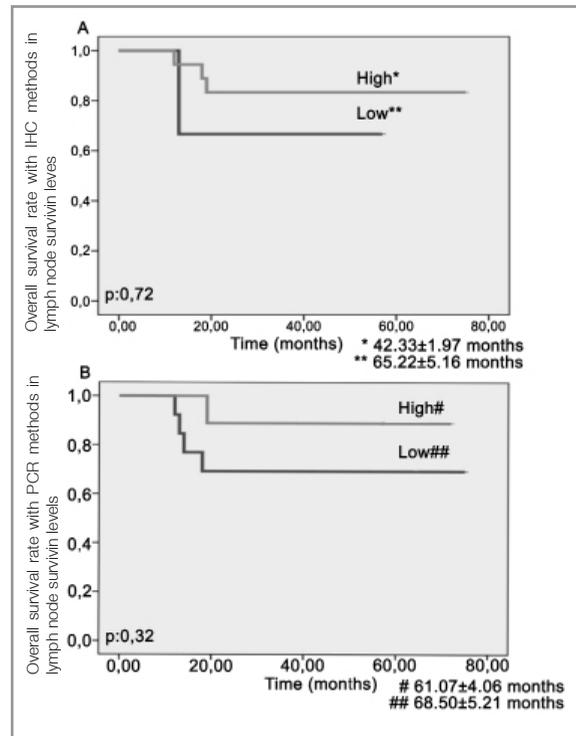

Figure 2. (A) Overall survival rate with IHC methods in lymph node survivin leves; **(B)** Overall survival rate with PCR methods in lymph node survivin levels

DISCUSSION

Survivin is expressed in embrionic cells and malignant cells, but it is not expressed in mature-differentiated cells.[19] The relationship between survivin, which is an anti-apoptotic protein, and a number of cancers were documented. It may show different functions according to the locations. Survivin, which is a member of IAP family inhibits apoptosis by inhibiting caspase 3 and 7.[20]

When the level of expression is evaluated, more survivin expression was reported in the study by Falleni et al. in patients with squamous cell lung cancer in comparison with adenocarcinoma and large cell subtypes.[21] In the studies by Sun, Huang no differences in expression were found between histological subtypes.[22,23]

Its prognostic value was assessed but clinicopathological correlations were not examined in any studies.[24-30] In terms of data on its prognostic significance, there are studies in which it was found to be a marker of poor prognosis, while there are other studies which have found the contrary.[25-27] Also,

there are no studies in the literature investigating the relationship between metastatic lymph node and clinical characteristics and survival in squamous cell subtype. Small groups of patients with squamous cell and adenocarcinoma were examined in these studies. A total of 225 patients with adenocarcinoma and 148 patients with squamous cell carcinoma were evaluated in the study by Sun et al, which may be considered as the most extensive investigation, and prognostic significance was not found in patients with squamous cell carcinoma.[22] There are no studies in which metastasis was investigated along with the tumor tissue. According to our study, it may be stated that it is one of the factors that contribute to progression. In terms of survival, the inverse relationship between survivin levels and survival in lymph node metastasis may provide an important clue for us, although it did not reach statistical significance. Along with data showing that survivin m-RNA levels may be effective in predicting response to anti-EGFR agents, there is also data that it may be effective in over-

coming resistance to therapy with tyrosine kinase inhibitors.[15,31]

It is also not clear how survivin levels should be evaluated. In a literature evaluation, values between 10-50% were found to be reported in studies with IHC method.[32] Furthermore, the threshold value with PCR method in the literature is different in each study.[33,34] A correlation between IHC and RT-PCR was not found in our study. But a conclusion may be reached with large-scale studies evaluating both methods together.

When racial differences are considered, it is hard to reach a conclusion, as there are no studies evaluating the relationship between survivin and squamous cell lung cancer in our society. Another important problem preventing conclusions in other racial groups is the larger number of studies on Asian populations.

The limitations of our study include the absence of control groups, the small sample size. Limitations in labour and economic conditions prevented us to overcome these limitations.

CONCLUSION

Survivin expression increases during the metastatic process and shows a negative correlation with OS. Higher survivin levels in lymph nodes detected by IHC were associated with shorter survival. An association was not found between survivin levels in tumor and lymph nodes and survival. Further larger studies done with larger numbers of patients are required in order to evaluate its effects in our society. Due to heterogeneity in the studies, more homogenous data are needed to assess survivin in squamous lung cancer. Also, combinations with targeted agents and immunotherapy may contribute in the prevention of resistance, and we believe that detailed investigations on this issue in the future may provide an effective strategy in increasing the probability of cure.

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