

Expression of Caspase-9 in Early and Locally Advanced Stage Squamous Cell Lung Cancer

¹Burcu YAPAR TAŞKÖYLÜ^a, ¹Aydın DEMİRAY^b, ¹Atike Gökçen DEMİRAY^a,
¹Gamze GÖKÖZ DOĞU^a, ¹Arzu YAREN^a, ¹Serkan DEĞİRMENCİOĞLU^a,
¹Nail ÖZHAN^c, ¹Canan KARAN^a, ¹Umut ÇAKIROĞLU^d, ¹Hakan AKÇA^b,
¹Ferda BİR^e, ¹Ahmet ERGİN^f

^aDepartment of Medical Oncology, Pamukkale University Faculty of Medicine, Denizli, TURKEY

^bDepartment of Medical Biology, Pamukkale University Faculty of Medicine, Denizli, TURKEY

^cClinic of Medical Oncology, Kayseri City Hospital, Kayseri, TURKEY

^dClinic of Medical Oncology, Van Training and Research Hospital, Van, TURKEY

^eDepartment of Pathology, Pamukkale University Faculty of Medicine, Denizli, TURKEY

^fDepartment of Social Pediatrics, Pamukkale University Faculty of Medicine, Denizli, TURKEY

ABSTRACT Objective: Apoptosis plays a critical role in carcinogenesis. Intracellular proteases known as caspase-9 directly or indirectly play roles inactivating apoptosis. In this study, we aimed to investigate the effect of caspase-9 level in lung squamous cancer tumor tissues on lymph node metastasis, clinicopathologic parameters and prognosis. **Material and Methods:** The study included 50 patients diagnosed with squamous cell lung cancer. The caspase-9 level in the tumor and metastatic lymph nodes was assessed using real time-polymerase chain reaction (RT-PCR) methods. **Results:** The median age of the patients was 61 years (range, 54-68 years). All patients were men and smokers. The average duration of follow-up was 50.26±39.21 months, and progression-free survival was 26.34±19.50 months. Among 50 patients, 22 (44%) had lymph node metastasis. The caspase-9 level was studied in tumors with and without lymph node metastasis. Moreover, the caspase-9 levels in tumors with lymph node metastasis were statistically and significantly lower than tumors without lymph node metastasis ($p < 0.001$). The caspase-9 level decreased as the stage increased ($p < 0.001$). In the presence of angiolymphatic invasion and poorly differentiated tumor tissue, a low level of caspase-9 was identified. **Conclusion:** The caspase-9 level decreased with increasing stage and the presence of lymph node metastasis. No statistically significant association was detected between caspase-9 and survival. A literature review revealed that no studies have evaluated the correlation between caspase-9 in the squamous cell subtype, clinicopathological parameters, and survival. Extensive studies involving a significant number of patients are required to determine the prognostic and predictive significance of the caspase-9 level in our population.

Keywords: Lung neoplasm; apoptosis; caspase 9

Lung cancer continues to be the leading cause of cancer-related deaths.¹ Only 15% of all patients with lung cancer have more than five years of survival after diagnosis.² Cytotoxic chemotherapy is the standard first-line therapy for patients with squamous cell lung cancer. More recent efforts have been made to identify novel biomarkers for the prediction of prognosis and response to chemotherapy.

Intrinsic and extrinsic apoptotic pathways use caspase steps. In the intrinsic pathway, Bax and Bak are

activated by various apoptotic stimuli through DNA damage or cellular signals. The activated Bax and Bak initiate mitochondria-dependent apoptotic pathway by inducing pore formation in the mitochondrial membrane, leading to the release of cytochrome c and other factors that form an apoptosome complex. Apoptosome activates caspase-9. Activated caspase-9 cleaves procaspase-3 to generate the active caspase-3 that leads to DNA fragmentation. The extrinsic pathway is activated when a death ligand, such as FasL or tumor

Correspondence: Burcu YAPAR TAŞKÖYLÜ

^aDepartment of Medical Oncology, Pamukkale University Faculty of Medicine, Denizli, TURKEY

E-mail: drburcuypar@gmail.com

Peer review under responsibility of Journal of Oncological Sciences.

Received: 31 May 2020

Received in revised form: 28 Aug 2020

Accepted: 02 Sep 2020

Available online: 29 Sep 2020

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necrosis factor- α interacts with cell surface receptors. These pathways form active caspase-8 from inactive procaspase-8. Caspase-8 activates caspase-3. Caspase-3 leads to DNA fragmentation again through caspase-activated DNase activation.^{3,4}

Caspase-9 can be activated using two methods. Apaf-1 activates caspase-9 by interacting with the CARD (Caspase Activation and Recruitment Domain) part of caspase-9 or activating the dimerization of the apoptosome.⁵⁻⁷

Molecular events involved in caspase-9 activation and inhibition may affect the response to chemotherapy drugs in non-small cell lung cancer cells.^{8,9} Moreover, caspase-9 plays a role in degenerative and developmental disorders. One study showed that a decreased level of caspase-9 affects muscle differentiation in the musculoskeletal system.¹⁰ In another study, the direct effect of caspase-9 as an apoptotic factor was demonstrated through neural cell death.¹¹

Studies are lacking in the literature, which evaluated the correlation between caspase-9 in the squamous cell cancer and clinicopathological parameters and survival. This study investigated whether the level of caspase-9 in tumor tissue affects the clinicopathological parameters and survival.

MATERIAL AND METHODS

We collected data from 50 patients with squamous cell lung carcinoma who underwent surgery and did not receive neoadjuvant chemotherapy or radiotherapy between 2011 and 2018. The patients were followed by the Department of Medical Oncology. The patient data on demographic data, tumor types, and chemotherapies received were obtained from the hospital information system. The study was conducted according to the Declaration of Helsinki and approved by the Pamukkale University Ethics Committee (Ethics Committee approval number: 2019/55821).

Determination of caspase-9 expression using reverse transcriptase-polymerase chain reaction (RT-PCR) method. This study obtained paraffin-embedded tissues of 50 patients with squamous cell lung carcinoma who attended the Medical Oncology Department, Pamukkale University, Denizli, between 2011 and 2014. RNA was extracted from paraffin-embedded tissues us-

ing the Qiamp DNA FFPE Tissue kit (Qiagen, Cat: 73504, Hilden, Germany). The purity of RNA was determined spectrophotometrically, and the samples were stored at -20 °C. cDNA was obtained from total RNA using IpsogenRT kit (Qiagen, Cat: 679923, Hilden, Germany). The mRNA expression level of caspase-9 was then measured using a LightCycler480 machine from primer Roche Applied Science (Roche, Basel, Switzerland). Data were analyzed using the $\Delta\Delta C_t$ method.

STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS version 23.0 (IBM, Chicago, IL, USA). We used Mann-Whitney U and Spearman's correlation tests for all clinical parameters. Overall and progression-free survivals were calculated using the Kaplan-Meier test. A *p*-value of <0.05 was considered statistically significant.

RESULTS

The median age of the patients was 61 years (range, 54-68 years). All patients had a history of smoking. The average duration of smoking was 29 months (range, 20-38 years). Among the 50 patients, nine (18%) had a history of cancer in the family, 28 (56%) had comorbidities, 22 (44%) had undergone adjuvant chemotherapy, and three (6%) had undergone adjuvant radiotherapy. The mean duration of follow-up of the patients was 50.26±39.21 months. The mean disease-free survival was 26.34±19.50 months. Moreover, 29 (58%) had a lobectomy, 20 (40%) had a pneumonectomy, and one (2%) had a wedge resection. The morphology was poorly differentiated in 38 patients and moderately well-differentiated in 12 patients. The tumor diameter ranged from 1 cm to 10 cm, with a mean diameter of 4.01±2.09 cm. Furthermore, 28 (56%) patients were identified as having angiolymphatic invasion, and 22 (44%) had lymph node metastasis. Moreover, 33 (66%) tumors were peripherally, and 17 (34%) were centrally located. In addition, 38 (78%) patients died during follow-up. The histopathological characteristics of the patients are presented in Table 1. The level of caspase-9 was studied in tumors with and without lymph node metastasis, which was 6.36±11.70 and 50.89±29.64, respectively. The caspase-9 level in tumors with lymph node metastasis was statistically and significantly lower than in those without lymph

TABLE 1: Clinicopathological and demographic characteristics of the patients.

Parameter	Number of patients(%)	Mean±SD
Age (years)	-	61.94±7.61
Gender		
Male	50 (100)	
Female	0 (0)	
History of smoking		
Yes	50 (100)	
No	0 (0)	
Duration of smoking (years)	-	29.60±8.91
Family history of cancer		
Yes	9 (18)	
No	41 (82)	
Comorbidities		
Yes	28 (56)	
No	22 (44)	
Type of operation		
Lobectomy	29 (58)	
Pneumonectomy	20 (40)	
Wedge resection	1 (2)	
Patients with lymphnode metastasis		
Yes	22 (44)	
No	28 (56)	
Stage		
1-2	28 (56)	
3	22 (44)	
Tumor Localization		
Peripheral	33 (66)	
Central	17 (34)	
Tumor Differentiation		
Low	35 (76)	
Moderate-well	15 (24)	
Angiolymphatic invasion		
Yes	28 (56)	
No	22 (44)	
Surgical margins		
Negative	47 (97)	
Positive	3 (9)	
Adjuvant chemotherapy		
Received	22 (44)	
Not received	28 (56)	
Adjuvant radiotherapy		
Received	3 (6)	
Not received	47 (94)	
Metastasis		
Yes	9 (18)	
No	41 (82)	
Progression-free survival (months)	-	26.34±19.50
Mean Overall Survival (months)	-	50.26±39.21
Caspase-9 level in tumor via PCR	-	31.30±28.27
Caspase-9 level in lymphnode via PCR	-	28.36±20.75

node metastasis ($p<0.001$). Table 2 shows no significant association among the caspase-9 level in the tumor tissue with lymph node metastasis, the presence of angiolymphatic invasion, differentiation, and metastasis. The caspase-9 level decreased as the stage increased ($p<0.001$), as shown in Figure 1. Caspase-9 was numerically lower in tumors with angiolymphatic invasion than in those without; however, the difference was not statistically significant. Similarly, caspase-9 was numerically lower in poorly differentiated tumors than moderately and well-differentiated tumors; however, the difference was not statistically significant.

DISCUSSION

Apoptosis is a process in which the cell enables several metabolic and physiological processes for self-destruction. A cell receiving an apoptosis signal moves away from its position, disconnects from its adjacent cells, and shrinks and gains a pyknotic appearance, with chromatin becoming dense. Caspase-9 plays a role in activating apoptosis.

TABLE 2: Effect of clinicopathological parameters on caspase-9 level in tumors

Factors	n	Tumor (RT-PCR)	
		Mean±SD	p-value
Age (years)			
≤65	33	33.82±35.52	0.75
>65	17	26.40±25.04	
Lymph node metastasis			
Yes	22	6.36±11.70	<0.001
No	28	50.89±29.64	
Tumor Differentiation			
Low	15	28.98±32.14	0.23
Moderately-well	38	34.90±31.78	
Angiolymphatic invasion			
Yes	12	26.90±32.72	0.072
No	29	36.89±31.54	
Metastasis			
Yes	9	31.45±25.07	0.85
No	41	31.26±33.91	
Stage			
1	31	42.59±31.44	<0.001
2	14	15.72±28.41	
3	5	4.90±3.56	

*Analyzed with univariate analysis, a Mann-Whitney U test and a Kruskal-Wallis test. * P<0.05 was considered statistically significant.

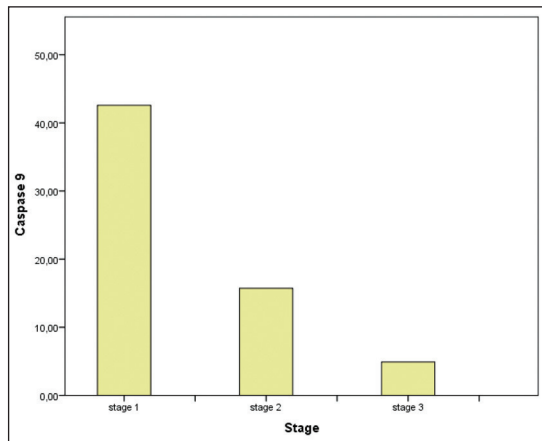


FIGURE 1: Graphic showing the tumor caspase-9 level and stage of association.

The results of this study showed that there is a statistically significant association between the tissue level of caspase-9 and lymph node metastasis. Ercan et al. also demonstrated lower levels of caspase-9 in tumors with lymph node metastasis.¹²

We observed a statistically significant association between stage and the tissue level of caspase-9. Yılmaz et al. identified an association between the CASP-9 (rs1052576) TT genotype and stage in prostate cancer.¹³

Our study results revealed that caspase-9 was numerically lower in poorly differentiated tumors (25.49 ± 17.42) than moderate and well-differentiated ones (41.30 ± 39.39); however, the difference was not statistically significant. Shen et al. investigated caspase-9 levels using RT-PCR in patients with stage II colon cancer. They found lower caspase-9 levels in poorly differentiated tumors than well-differentiated tumors.¹⁴ In this study, caspase-9 was numerically lower in tumors with angiolymphatic invasion (28.98 ± 32.14) than in those without (36.89 ± 31.54); however, the difference was not statistically significant.

Caspase 9 plays a role in carcinogenesis in several tumor types. Previous studies have reported that caspase-9 polymorphism affects caspase-9 levels and poses a susceptibility to lung cancer.^{15,16} Asadi et al. investigated caspase-9 levels in colorectal cancer and reported that caspase gene mRNA expression has an effect on the development of colorectal carcinoma.¹⁷ On a literature review of studies examining caspase-9 levels in patients with breast cancer, Theodoropoulos et al. reported that caspase-9 rs4645978 and rs4645981

gene polymorphisms posed a susceptibility to breast cancer.¹⁸ Zhang et al. found that miR-224 decreased caspase-9 levels and induced carcinogenesis in patients with triple-negative breast cancer.¹⁹ Özdoğan et al. reported a protective effect of the presence of CASP-9 [rs1052576] mutant A allele in patients with brain tumors.²⁰ Marques et al. examined caspase-9 and survival in renal cell carcinoma and found an association between the CASP9+83CT/TT genotype and the development of renal cell carcinoma.²¹

Caspase 9 has a predictive role in response to treatments. Sharifi et al. showed that caspase-9 levels decreased in patients with breast cancer who were resistant to paclitaxel.²² Kemper et al. observed that apoptosis increased after administering inducible caspase-9 into colorectal cancer stem cells and inducible caspase-9 enhanced apoptosis and tumor regression in patients resistant to 5-fluorouracil, as caspase-9-induced apoptosis does not use the mitochondrial pathway when compared with 5-fluorouracil-induced apoptosis.²³ Kuwahara et al. reported that the inhibition of caspase-9 activity resulted in cisplatin resistance in patients with head and neck carcinoma.²⁴ Iwani et al. demonstrated that miR-96-5p inhibited apoptosis by targeting CASP9.²⁵

No statistically significant association was observed between caspase-9 level and survival, although we believed that statistical significance could not be achieved because of the low number of patients. Strater et al. reported that high levels of caspase-9 are associated with poor prognosis in colorectal cancer.²⁶ Terlizzi et al. reported an association between elevated levels of caspase-4 in tissues and decreased overall survival in an analysis of caspase-4 levels using the enzyme-linked immunosorbent assay.²⁷ Liu et al. established an association between caspase-8 polymorphism. It decreased overall survival in a study of 555 patients diagnosed with advanced-stage adenocarcinoma in which caspase-8 polymorphism was analyzed in peripheral blood samples.²⁸ Theodoropoulos et al. examined caspase-8 and caspase-9 gene polymorphisms in patients with colon cancer and revealed better prognosis in patients with the CASP9-1263 GG genotype.²⁹

CONCLUSION

Caspase-9 expression differs in patients with lymph node metastasis and stage. The caspase-9 level decreased with

increasing stage and the presence of lymph node metastasis. In addition, a numerically lower level of caspase-9 was noted in the presence of angiolymphatic invasion and poorly differentiated tumors. No association was found between caspase-9 and survival. This is the first study in the literature to examine caspase-9 in tumors and lymph nodes using RT-PCR. Further extensive studies involving a significant number of patients are required to evaluate its effects on our population.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that provides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: Burcu Yapar Taşköylü; **Design:** Gamze Gököz Doğu; **Control/Supervision:** Arzu Yaren; **Data Collection and/or Processing:** Atike Gökçen Demiray; **Analysis and/or Interpretation:** Aydın Demiray, Ahmet Ergin; **Literature Review:** Nail Özhan, Canan Karan; **Writing the Article:** Serkan Değirmencioglu, Umut Çakıroğlu; **Writing the Article:** Serkan Değirmencioglu, Umut Çakıroğlu; **Critical Review:** Hakan Akça, Ferda Bir; **References and Fundings:** Burcu Yapar Taşköylü; **Materials:** Burcu Yapar Taşköylü.

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