Research Article / Araştırma Makalesi
doi: 10.18678/dtfd.1294988

Duzce Med J, 2023;25(2):158-166
Düzce Tıp Fak Derg, 2023;25(2):158-166

The Expression of Caspase-3 and GRIM-19 in Non-mucinous Lung Adenocarcinoma and Their Clinicopathologic Significance

Müsinöz Olmayan Akciğer Adenokarsinomlarında Caspase-3 ve GRIM-19 Ekspresyonu ile Bu Proteinlerin Klinikopatolojik Önemi

Alev OK ATILGAN[1]
0000-0001-8595-8880
Merih TEPEOĞLU[1]
0000-0002-9894-8005
Eda YILMAZ AKÇAY[1]
0000-0001-6831-9585
Leyla HASANALİYEVA[1]
0000-0002-6545-0286
Dalokay KILIÇ[2]
0000-0001-7813-5317
Handan ÖZDEMİR[1]
0000-0002-7528-3557

[1]Department of Pathology, Başkent University Faculty of Medicine, Ankara, Türkiye

[2]Department of Thoracic Surgery, Başkent University Faculty of Medicine, Ankara, Türkiye

Corresponding Author
Sorumlu Yazar
Alev OK ATILGAN
aokatilgan@gmail.com

Received / Geliş Tarihi : 10.05.2023
Accepted / Kabul Tarihi : 23.07.2023
Available Online / Çevrimiçi Yayın Tarihi : 10.08.2023

ABSTRACT

Aim: The current study aimed to investigate apoptotic proteins such as caspase-3 and GRIM-19 protein expression in non-mucinous lung adenocarcinomas and their clinicopathologic significance.
Material and Methods: This study was performed on 81 patients diagnosed with non-mucinous lung adenocarcinoma between January 1, 2010, and June 1, 2020. Immunohistochemical analysis was performed to examine the expressions of caspase-3 and GRIM-19, and the association between these proteins and clinicopathological parameters was investigated.
Results: Caspase-3 nuclear positivity was more common in high-grade non-mucinous lung adenocarcinomas (p<0.001). Caspase-3 cytoplasmic expression was stronger in tumors with advanced-stage (p=0.021) and lymph node metastases (p=0.020). GRIM-19 expression was low in tumors with high-grade non-mucinous lung adenocarcinomas (p=0.002), and tumors with lymphovascular invasion (p=0.021). The median follow-up time was 31.7 (range, 1-145 months. The overall 5-year survival rate of patients with low and high GRIM-19 expression tumors was 48% and 92%, respectively. GRIM-19 expression significantly affected the 5-year overall survival rate (p=0.008), but not the 5-year disease-free survival rate (p=0.368).
Conclusion: We revealed a significant association between caspase-3 and GRIM-19 expressions and poor clinicopathologic features and prognosis. For the first time in the literature, we revealed an association between low GRIM-19 expression and worse clinical outcomes in patients with non-mucinous lung adenocarcinoma. Caspase-3 and GRIM-19 may become potential therapeutic targets and novel potential predictive biomarkers for non-mucinous lung adenocarcinoma patients.
Keywords: Apoptosis; Caspase-3; GRIM-19; lung adenocarcinoma.

ÖZ

Amaç: Bu çalışmada, müsinöz olmayan akciğer adenokarsinomlarında, apoptotik protein olarak bilinen caspase-3 ve GRIM-19 protein ekspresyonu ve bu proteinlerin klinikopatolojik öneminin araştırılması amaçlandı.
Gereç ve Yöntemler: Bu çalışma, 1 Ocak 2010 ile 1 Haziran 2020 tarihleri arasında müsinöz olmayan akciğer adenokarsinomu tanısı alan 81 hasta üzerinde gerçekleştirildi. Caspase-3 ve GRIM-19 ekspresyonlarını incelemek için immünohistokimyasal analiz yapıldı ve bu proteinler ile klinikopatolojik parametreler arasındaki ilişki araştırıldı.
Bulgular: Caspase-3 nükleer pozitifliği yüksek dereceli müsinöz olmayan akciğer adenokarsinomlarında daha yaygın bulundu (p<0,001). Caspase-3 sitoplazmik ekspresyonu ileri evre (p=0,021) ve lenf nodu metastazı (p=0,020) olan tümörlerde daha güçlü saptandı. GRIM-19 ekspresyonu, yüksek dereceli müsinöz olmayan akciğer adenokarsinomlu tümörlerde (p=0,002) ve lenfovasküler invazyonlu tümörlerde (p=0,021) düşük idi. Ortanca takip süresi 31,7 (aralık, 1-145) ay idi. Düşük ve yüksek GRIM-19 ekspresyonlu tümörlere sahip hastaların 5 yıllık genel sağkalım oranı sırasıyla %48 ve %92 idi. GRIM-19 ekspresyonu genel 5 yıllık sağkalım oranı üzerinde anlamlı bir etkisi olduğu (p=0,008), ancak 5 yıllık hastalıksız sağkalım oranı üzerinde anlamlı bir etkisi olmadığı (p=0,368) saptandı.
Sonuç: Caspase-3 ve GRIM-19 ekspresyonu ile kötü klinikoptolojik özellikler ve prognoz arasında anlamlı bir ilişki olduğunu gösterdik. Literatürde ilk kez, düşük GRIM-19 ekspresyonunun müsinöz olmayan akciğer adenokarsinomunda daha kötü bir klinik gidişle ilişkili olduğunu ortaya koyduk. Caspase-3 ve GRIM-19 müsinöz olmayan akciğer adenokarsinomu hastaları için yeni potansiyel prognostik biyobelirteçlerin yanı sıra potansiyel terapötik hedefler haline gelebilir.
Anahtar kelimeler: Apoptoz; Caspase-3; GRIM-19; akciğer adenokarsinomu.

INTRODUCTION

Invasive non-mucinous adenocarcinoma is the most common subtype of lung carcinoma and is responsible for the highest number of cancer-related fatalities globally (1,2). The aggressive nature of lung adenocarcinomas persists despite significant progress in treatment alternatives, attributed to mutations in multiple oncogenes, tumor suppressor genes, and apoptotic proteins (3).

Initiation of apoptosis involves a series of sequential steps triggered by the activation of a group of cysteine proteases known as "caspases" (4). Caspase-3, a well-known member of the caspase enzyme family, assumes a crucial role in both the intrinsic and extrinsic pathways of apoptosis (4). The extrinsic pathway primarily involves death receptor proteins located on the cytoplasmic membrane, whereas the intrinsic pathway is initiated by a disruption of mitochondrial membrane permeability, leading to the release of cytochrome c from the intermembranous space of mitochondria into the cytoplasm. Subsequently, cytochrome c activates caspase-3, initiating a cascade in the intrinsic apoptotic pathway (4,5). In addition to its apoptotic influence, caspase-3 released from apoptotic cells exerts an impact on the proliferation, differentiation, and survival of neighboring malignant or normal cells (4).

On the other hand, Liu et al. (6) demonstrated that mitochondrial permeability changes lead to spontaneous cytochrome c leakage and sublethal caspase-3 activation specifically in cancer cells, rather than in normal cells. Sublethal caspase-3 activation does not cause apoptosis but leads to spontaneous DNA double-strand breaks, chromosomal instability, and gene mutations, and promotes malignant transformation (6). Thus, caspase-3 is crucial for cell maintenance. Previous studies have demonstrated caspase-3 expression in some malignant tumors, such as breast, colorectal, cervical, squamous cell carcinoma, and glioma (7). However, the literature currently lacks sufficient data on caspase-3 expression in lung adenocarcinoma and its impact on prognosis.

The interferon (IFN) family of cytokines is known for its effectiveness in antiviral and immune responses, as well as its antitumor effects. The primary effect of IFNs is the elimination of infected or neoplastic cells by inducing cell cycle inhibition or apoptosis. Retinoic acid (RA), a natural metabolite of vitamin A, is involved in cell growth, differentiation, and embryogenesis (8). The combination therapy of IFN and RA exhibits a synergistic effect, leading to more effective inhibition of tumor growth. Previous studies have revealed enhanced tumor growth inhibition with IFN/RA combination therapy (8-10). GRIM-19 is involved in the IFN-β/RA-induced cell death pathway and has been shown to synergistically suppress cell cycle progression (8-10) and exhibit tumor suppressor effects (11). Loss or significant reduction of GRIM-19 expression has been reported in various malignancies, such as cervical, ovarian, kidney, colorectal, and hepatocellular carcinoma (10,12). However, it has been reported that GRIM-19 overexpression induces apoptosis in the human breast carcinoma cell line MCF-7, and gastric cancer cells, and suppresses hepatocellular carcinoma growth (13,14). A molecular study conducted by Wang et al. (15) revealed a decrease in GRIM-19 RNA and protein levels, and tumor cell growth was suppressed by GRIM-19 overexpression, promoting tumor cell apoptosis in vivo and in vitro in lung adenocarcinoma. Despite this, the precise relationship between GRIM-19 expression, clinicopathological features, and its influence on tumor progression remains incompletely understood.

This study aimed twofold: firstly, to evaluate the expression of caspase-3 and GRIM-19 in invasive non-mucinous lung adenocarcinomas, and secondly, to explore the association between their expression and clinicopathological parameters, potentially offering valuable prognostic insights.

MATERIAL AND METHODS

The Clinical Research Ethics Committee of Baskent University (Project no: KA23/162; Date: 27 April 2023) approved the current study which was compatible with the ethical guidelines of the Declaration of Helsinki. This study included 81 patients diagnosed with non-mucinous lung adenocarcinoma after lobectomy or pneumonectomy between January 1, 2010, and June 1, 2020. Patients receiving preoperative chemotherapy and/or radiotherapy treatment and patients with mucinous adenocarcinoma were excluded. Two pathologists (MT, EYA) re-evaluated all histopathological slides. Clinical follow-up findings of all patients were obtained from hospital records. The classification of tumors was carried out according to the diagnostic criteria established by the World Health Organization (WHO) in 2021, and the staging was performed using the American Joint Committee on Cancer (AJCC) 8th edition (1).

Tissue microarray (TMA) blocks were prepared by removing tissue from two representative foci involving different areas with a diameter of 2 mm. Immunohistochemically, rabbit polyclonal Caspase-3 antibody (IgG isotype, 1:100; Genetex) and rabbit monoclonal GRIM-19 antibody (clone EPR4471 (2), 1:200; Epitomics) were applied on the 4μm-thick slices of TMA blocks with Dako Omnis system and are stained with EnVision FLEX staining kits. Appropriate positive controls were used.

Two pathologists (AOA, MT) independently evaluated the immunohistochemistry-stained slides. The nuclear caspase-3 immunoreactivity was assessed semiquantitatively by determining the percentage of cells exhibiting positive staining: <10% staining was classified as negative, while >10% staining was classified as positive (16). For the cytoplasmic staining intensity of caspase-3, the tumor was graded as follows: 1 for mild expression, 2 for moderate expression, and 3 for strong expression (17). GRIM-19 expression in the cytoplasm, with or without a nucleus, was also evaluated semiquantitatively. The intensity was scored as 0 for no staining, 1 for mild staining, 2 for moderate staining, and 3 for strong staining. The percentage of positive tumor cells was scored from 0 to 100%. An h-score was calculated by multiplying the intensity score and the percentage of staining. An h-score ≤120 indicated low expression, while an h-score >120 indicated high expression (18).

Caspase-3 and GRIM-19 expression in the non-mucinous lung adenocarcinoma tissues is illustrated in Figure 1 and Figure 2, respectively.

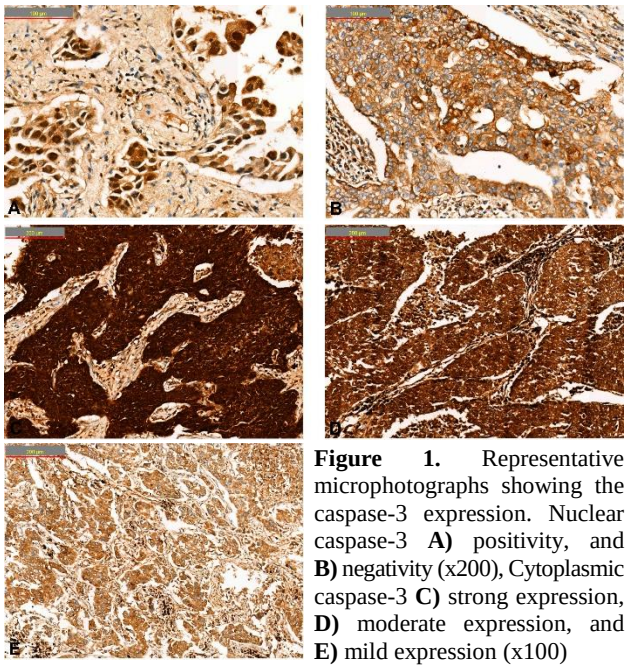

Figure 1. Representative microphotographs showing the caspase-3 expression. Nuclear caspase-3 **A)** positivity, and **B)** negativity (x200), Cytoplasmic caspase-3 **C)** strong expression, **D)** moderate expression, and **E)** mild expression (x100)

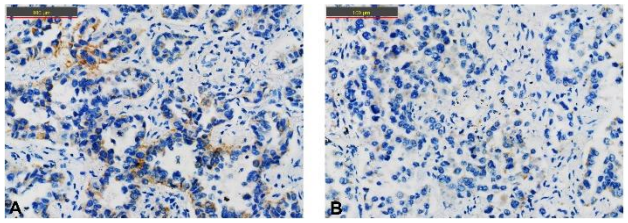

Figure 2. Representative microphotographs showing the GRIM-19 expression. **A)** high, and **B)** low GRIM-19 expression (x200)

Table 1. Clinicopathologic characteristics of patients with non-mucinous lung adenocarcinoma

Age (years), median (range)	65 (36-85)
Age, n (%)	
<65 years	39 (48.1)
≥65 years	42 (51.9)
Gender, n (%)	
Female	20 (24.7)
Male	61 (75.3)
Smoking status, n (%)	
Current smoker	38 (46.9)
Ex-Smoker	25 (30.9)
Never Smoker	18 (22.2)
Surgery, n (%)	
Lobectomy	71 (87.7)
Pneumonectomy	10 (12.3)
Tumor size (cm), median (range)	2.4 (0.8-11.5)
Tumor size, n (%)	
<2.5 cm	40 (49.4)
≥2.5 cm	41 (50.6)
Histology, n (%)	
Lepidic	4 (4.9)
Aciner	46 (56.8)
Papillary	8 (9.9)
Micropapillary	3 (3.7)
Solid	20 (24.7)
Stage, n (%)	
Stage I	44 (54.3)
Stage II	10 (12.3)
Stage III	24 (29.6)
Stage IV	3 (3.7)
Lymphovascular invasion, n (%)	
Absent	36 (44.4)
Present	45 (55.6)
Lymph node metastases, n (%)	
Absent	50 (61.7)
Present	31 (38.3)
Perineural invasion, n (%)	
Absent	72 (88.9)
Present	9 (11.1)
Necrosis, n (%)	
Absent	39 (48.1)
Present	42 (51.9)
Visseral pleura involvement, n (%)	
Absent	34 (42.0)
Present	47 (58.0)
Recurrence, n (%)	
No recurrence	70 (86.4)
Recurrence	11 (13.6)
Follow-up status, n (%)	
Dead of disease	30 (37.0)
Alive with recurrent disease	3 (3.7)
Alive with no evidence of disease	46 (56.8)
Dead of nondisease	2 (2.5)

Statistical Analysis

Statistical analyses were made with the IBM SPSS v.25 program. Descriptive analyzes were presented using median and minimum-maximum values. Analytical methods (Kolmogorov-Smirnov test) were used to determine whether the variables were normally distributed. Since normal distribution could not obtain, non-parametric tests were performed. The Pearson chi-square or Fisher's exact test was used to compare the qualitative variables and represented by numbers and percentages. The Univariate Kaplan-Meier method was used to estimate overall survival (OS) and disease-free survival (DFS) rates and results were compared using the long-rank test. Statistical significance was considered only for p-values below 0.05.

RESULTS

Among the 81 patients enrolled in the study, 61 (75.3%) were male, while 20 (24.7%) were female. The median age was 65 (range, 36-85) years. Tumor diameters ranged from 0.8 cm to 11.5 cm, with a median of 2.4 cm. Among the tumors, 4 (4.9%) were grade 1 (all showed a lepidic pattern), 54 (66.7%) were grade 2 (46 showed an acinar, 3 showed a papillary), and 23 (28.4%) were grade 3 (3 showing a micropapillary, 20 showed a solid pattern). 44 (54.3%) tumors were classified as stage I, 10 (12.3%) as stage II, 24 (29.6%) as stage III, and 3 (3.7%) as stage IV disease. Early-stage tumors included stages I and II, while advanced-stage tumors included stages III and IV. The summary of the clinicopathological characteristics of the study cases was presented in Table 1.

Tumors showed staining in the tumor cells from 0% to 100% and varying intensities of cytoplasmic caspase-3 expression with or without nuclear expression. As shown in Table 2, 65 (80.2%) tumors exhibited caspase-3 nuclear positivity. Among the different histologic patterns, nuclear caspase-3 positivity was observed in none of the lepidic pattern adenocarcinomas, 41 (89.1%) of the acinar pattern, 4 (50%) of the papillary pattern, all (100%) of the micropapillary pattern, and 17 (85%) of the solid pattern tumors. Additionally, caspase-3 nuclear positivity was

found in none of 4 histologic grade 1 tumors, 45 (83.3%) of 54 histologic grade 2, and 20 (87%) of 23 histologic grade 3. Caspase-3 nuclear positivity demonstrated a significant association with tumor histology (p<0.001) and histologic grading (p<0.001). There were no significant associations observed between nuclear positivity of caspase-3 and age (p=0.469), gender (p=0.060), smoking status (p=0.208), tumor size (p=0.956), tumor stage (p=0.844), pT stage (p=0.979), lymphovascular invasion (p=0.289), lymph node metastases (p=0.519), perineural invasion (p=0.490), and necrosis (p=0.694).

Regarding caspase-3 cytoplasmic expression, 25 (30.9%) of 81 non-mucinous lung adenocarcinomas had mild expression, 40 (49.4%) had moderate, and 16 (19.8%) had strong expression. Among the early-stage tumors, 19 (35.2%) showed mild caspase-3 cytoplasmic expression, 29 (53.7%) showed moderate expression, and 6 (11.1%) showed strong expression. Among the advanced-stage tumors, 6 (22.2%) showed mild caspase-3 cytoplasmic expression, 11 (40.7%) showed moderate expression, and 10 (37.0%) showed strong expression. Caspase-3 cytoplasmic expression was stronger in advanced-stage compared to early-stage tumors, and a significant association was observed between caspase-3 cytoplasmic expression and tumor stage (p=0.021). Tumors with lymph node metastases exhibited stronger caspase-3 cytoplasmic expression compared to those without metastases (p=0.020). There were no significant associations observed between cytoplasmic expression of caspase-3 and age (p=0.583), gender (p=0.551), smoking status (p=0.598), tumor size (p=0.607), histology (p=0.731), histologic grade (p=0.345), pT stage (p=0.635), lymphovascular invasion (p=0.215), perineural invasion (p=0.595), and presence of necrosis (p=0.741).

For GRIM-19 expression, tumors displayed varying intensities of cytoplasmic expression without nuclear expression. 12 (14.8%) of 81 non-mucinous lung adenocarcinomas exhibited high GRIM-19 expression, and 69 (85.2%) exhibited low GRIM-19 expression. Low GRIM-19 expression was observed in 1 (25%) of the lepidic pattern adenocarcinomas, 42 (91.3%) of the acinar pattern, 6 (75%) of the papillary pattern, 2 (66.7%) of the micropapillary pattern, and 18 (90%) of the solid pattern tumors (p=0.005). Low GRIM-19 expression was observed in 1 (25%) of the histologic grade 1 tumors, 48 (88.9%) of the histologic grade 2 tumors, and 20 (87%) of the histologic grade 3 tumors. Tumors with higher histologic grades exhibited lower GRIM-19 expression compared to those with lower grades, indicating a significant association between GRIM-19 expression and histologic grading (p=0.002). Among the advanced-stage tumors, 26 (96.3%) showed low GRIM-19 expression, while among the early-stage tumors, 43 (79.6%) showed low expression, and a significant association was observed between GRIM-19 expression and tumor stage (p=0.047). Among tumors with lymphovascular invasion, 42 (93.3%) exhibited low GRIM-19 expression, while among tumors without lymphovascular invasion, 27 (75%) exhibited low expression, indicating a significant association between GRIM-19 expression and lymphovascular invasion (p=0.021). There were no significant associations observed between GRIM-19 expression and age (p=0.889), gender (p=0.452), smoking status (p=0.252), tumor size (p=0.502), pT stage (p=0.074), lymph node metastases (p=0.095), perineural invasion (p=0.740), and necrosis (p=0.444).

The median follow-up duration was 31.7 (range, 1-145) months. Out of the 81 patients, 30 (37.0%) died from carcinoma, while 2 (2.5%) patients died from other causes without evidence of recurrence and were considered censored. Three (3.7%) patients were still alive with the disease, and 46 (56.8%) were alive with no evidence of disease.

The 5-year OS rate showed a significant difference between patients with and without tumors exhibiting lymphovascular invasion (26% vs. 62%, p=0.009, Figure 3). The median overall survival time for patients with low GRIM-19 expression was 29.42 (range, 1-109) months, while for patients with high GRIM-19 expression, it was 44.35 (range, 14-145) months. The 5-year OS rate for patients with low GRIM-19 expression tumors was 48%, whereas, for patients with high GRIM-19 expression tumors, it was 92%. Univariate Kaplan-Meier/log-rank analyses showed that patients with tumors exhibiting low GRIM-19 expression had significantly lower 5-year OS rates compared to those with high GRIM-19 expression (p=0.008, Figure 4A). Nevertheless, there was no significant association between the 5-year OS rate and caspase-3 cytoplasmic expression (p=0.330, Figure 4B), caspase-3 nuclear expression (p=0.412, Figure 4C), and other clinicopathologic factors such as age (p=0.827), gender (p=0.429), smoking (p=0.738), tumor size (p=0.616), tumor histology (p=0.476), histologic grading (p=0.601), tumor stage (p=0.119), pT stage (p=0.076), lymph node metastases (p=0.353), perineural invasion (p=0.565), and presence of necrosis (p=0.169).

Only 11 (13.6%) experienced recurrence. The median time to recurrence was 21.74 (range, 4.9-41.7) months. The 5-year DFS rate showed no significant association with the GRIM-19 expression (p=0.368, Figure 5A), caspase-3 cytoplasmic expression (p=0.581, Figure 5B), caspase-3 nuclear expression (p=0.447, Figure 5C), and clinicopathologic factors such as age (p=0.785), gender (p=0.187), smoking (p=0.094), tumor size (p=0.090),

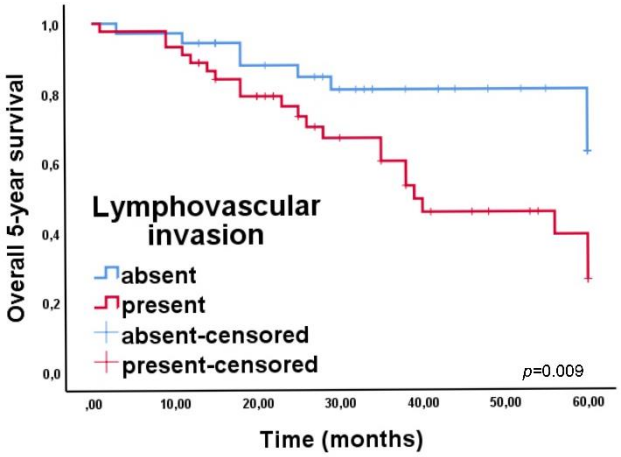

Figure 3. The Kaplan-Meier curves of lymphovascular invasion for 5-year overall survival rate. The 5-year OS rate of patients with tumors with lymphovascular invasion and no lymphovascular invasion was 26% and 62% (p=0.009)

Table 2. The association between caspase-3 and GRIM-19 expression and clinicopathologic features

	Age		**p**
	<65 years (n=39)	**≥65 years (n=42)**	
Caspase-3 nuclear expression, n (%)			
Negative	9 (23.1)	7 (16.7)	0.469
Positive	30 (76.9)	35 (83.3)	
Caspase-3 cytoplasmic expression, n (%)			
Mild	13 (33.3)	12 (28.6)	
Moderate	17 (43.6)	23 (54.8)	0.583
Strong	9 (23.1)	7 (16.7)	
GRIM-19 expression, n (%)			
Low	33 (84.6)	36 (85.7)	0.889
High	6 (15.4)	6 (14.3)	
	Gender		**p**
	Female (n=20)	**Male (n=61)**	
Caspase-3 nuclear expression, n (%)			
Negative	7 (35)	9 (14.8)	0.060
Positive	13 (65)	52 (85.2)	
Caspase-3 cytoplasmic expression, n (%)			
Mild	8 (40)	17 (27.9)	
Moderate	8 (40)	32 (52.5)	0.551
Strong	4 (20)	12 (19.7)	
GRIM-19 expression, n (%)			
Low	16 (80)	53 (86.9)	0.452
High	4 (20)	8 (13.1)	
	Smoking Status		**p**
	Current smoker (n=38)	**Ex-Smoker (n=25)**	**Never Smoker (n=18)**	
Caspase-3 nuclear expression, n (%)				
Negative	5 (13.2)	5 (20)	6 (33.3)	0.208
Positive	33 (86.8)	20 (80)	12 (66.7)	
Caspase-3 cytoplasmic expression, n (%)				
Mild	12 (31.6)	7 (28)	6 (33.3)	
Moderate	16 (42.1)	15 (60)	9 (50.0)	0.598
Strong	10 (26.3)	3 (12)	3 (16.7)	
GRIM-19 expression, n (%)				
Low	35 (92.1)	20 (80)	14 (77.8)	0.252
High	3 (7.9)	5 (20)	4 (22.2)	
	Tumor size		**p**
	<2.4 cm (n=40)	**≥2.4 cm (n=41)**	
Caspase-3 nuclear expression, n (%)			
Negative	8 (20)	8 (19.5)	0.956
Positive	32 (80)	33 (80.5)	
Caspase-3 cytoplasmic expression, n (%)			
Mild	11 (27.5)	14 (34.1)	
Moderate	22 (55.0)	18 (43.9)	0.607
Strong	7 (17.5)	9 (22.0)	
GRIM-19 expression, n (%)			
Low	33 (82.5)	36 (87.8)	0.502
High	7 (17.5)	5 (12.2)	
	Histology					**p**
	Lepidic (n=4)	**Aciner (n=46)**	**Papillary (n=8)**	**Micropapillary (n=3)**	**Solid (n=20)**	
Caspase-3 nuclear expression, n (%)						
Negative	4 (100)	5 (10.9)	4 (50)	0 (0)	3 (15)	**<0.001**
Positive	0 (0)	41 (89.1)	4 (50)	3 (100)	17 (85)	
Caspase-3 cytoplasmic expression, n (%)						
Mild	3 (75)	14 (30.4)	2 (25)	1 (33.3)	5 (25)	
Moderate	1 (25)	22 (47.8)	4 (50)	2 (66.7)	11 (55)	0.731
Strong	0 (0)	10 (21.7)	2 (25)	0 (0)	4 (20)	
GRIM-19 expression, n (%)						
Low	1 (25)	42 (91.3)	6 (75)	2 (66.7)	18 (90)	**0.005**
High	3 (75)	4 (8.7)	2 (25)	1 (33.3)	2 (10)	
	Histologic Grade			**p**
	Grade 1 (n=4)	**Grade 2 (n=54)**	**Grade 3 (n=23)**	
Caspase-3 nuclear expression, n (%)				
Negative	4 (100)	9 (16.7)	3 (13)	**<0.001**
Positive	0 (0)	45 (83.3)	20 (87)	
Caspase-3 cytoplasmic expression, n (%)				
Mild	3 (75)	16 (29.6)	6 (26.1)	
Moderate	1 (25)	26 (48.1)	13 (56.5)	0.345
Strong	0 (0)	12 (22.2)	4 (17.4)	
GRIM-19 expression, n (%)				
Low	1 (25)	48 (88.9)	20 (87)	**0.002**
High	3 (75)	6 (11.1)	3 (13)	

Table 2. (continued) The association between caspase-3 and GRIM-19 expression and clinicopathologic features

	Tumor Stage		**p**
	Early Stage (I-II) (n=54)	**Advanced Stage (III-IV) (n=27)**	
Caspase-3 nuclear expression, n (%)			
Negative	11 (20.4)	5 (18.5)	0.844
Positive	43 (79.6)	22 (81.5)	
Caspase-3 cytoplasmic expression, n (%)			
Mild	19 (35.2)	6 (22.2)	
Moderate	29 (53.7)	11 (40.7)	**0.021**
Strong	6 (11.1)	10 (37.0)	
GRIM-19 expression, n (%)			
Low	43 (79.6)	26 (96.3)	**0.047**
High	11 (20.4)	1 (3.7)	
	pT Stage		**p**
	pT1-2 (n=66)	**pT3-4 (n=15)**	
Caspase-3 nuclear expression, n (%)			
Negative	13 (19.7)	3 (20)	0.979
Positive	53 (80.3)	12 (80)	
Caspase-3 cytoplasmic expression, n (%)			
Mild	19 (28.8)	6 (40.0)	
Moderate	33 (50.0)	7 (46.7)	0.635
Strong	14 (21.2)	2 (13.3)	
GRIM-19 expression, n (%)			
Low	54 (81.8)	15 (100)	0.074
High	12 (18.2)	0 (0)	
	Lymphovascular Invasion		**p**
	Absent (n=36)	**Present (n=45)**	
Caspase-3 nuclear expression, n (%)			
Negative	9 (25)	7 (15.6)	0.289
Positive	27 (75)	38 (84.4)	
Caspase-3 cytoplasmic expression, n (%)			
Mild	12 (33.3)	13 (28.9)	
Moderate	20 (55.6)	20 (44.4)	0.215
Strong	4 (11.1)	12 (26.7)	
GRIM-19 expression, n (%)			
Low	27 (75)	42 (93.3)	**0.021**
High	9 (25)	3 (6.7)	
	Lymph Node Metastases		**p**
	Absent (n=50)	**Present (n=31)**	
Caspase-3 nuclear expression, n (%)			
Negative	11 (22)	5 (16.1)	0.519
Positive	39 (78)	26 (83.9)	
Caspase-3 cytoplasmic expression, n (%)			
Mild	17 (34)	8 (25.8)	
Moderate	28 (56)	12 (38.7)	**0.020**
Strong	5 (10)	11 (35.5)	
GRIM-19 expression, n (%)			
Low	40 (80)	29 (93.5)	0.095
High	10 (20)	2 (6.5)	
	Perineural Invasion		**p**
	Absent (n=72)	**Present (n=9)**	
Caspase-3 nuclear expression, n (%)			
Negative	15 (20.8)	1 (11.1)	0.490
Positive	57 (79.2)	8 (88.9)	
Caspase-3 cytoplasmic expression, n (%)			
Mild	21 (29.2)	4 (44.4)	
Moderate	36 (50.0)	4 (44.4)	0.595
Strong	15 (20.8)	1 (11.1)	
GRIM-19 expression, n (%)			
Low	61 (84.7)	8 (88.9)	0.740
High	11 (15.3)	1 (11.1)	
	Necrosis		**p**
	Absent (n=39)	**Present (n=42)**	
Caspase-3 nuclear expression, n (%)			
Negative	7 (17.9)	9 (21.4)	0.694
Positive	32 (82.1)	33 (78.6)	
Caspase-3 cytoplasmic expression, n (%)			
Mild	11 (28.2)	14 (33.3)	
Moderate	21 (53.8)	19 (45.2)	0.741
Strong	7 (17.9)	9 (21.4)	
GRIM-19 expression, n (%)			
Low	32 (82.1)	37 (88.1)	0.444
High	7 (17.9)	5 (11.9)	

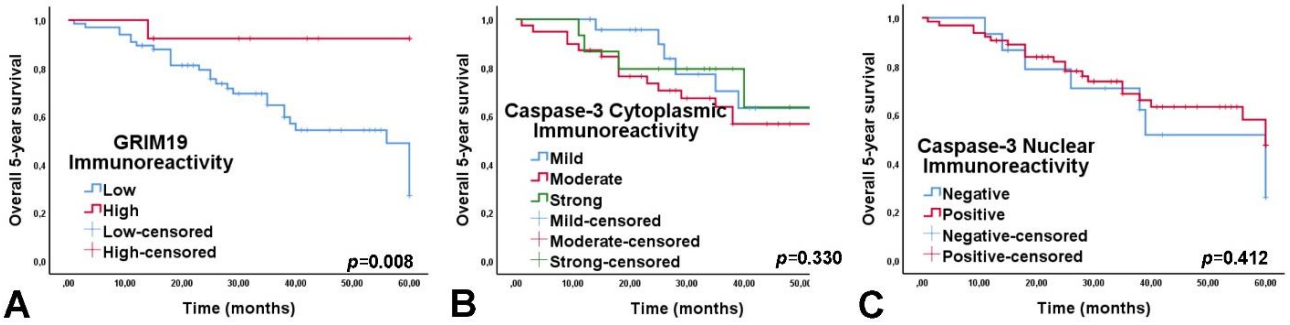

Figure 4. The Kaplan-Meier curves of GRIM-19 expression, caspase-3 cytoplasmic expression, and caspase-3 nuclear expression for 5-year overall survival. **A)** the 5-year OS rate of patients with a low GRIM-19 expression was significantly decreased compared with those with a high GRIM-19 expression (p=0.008), however, the 5-year OS rate showed no association with **B)** caspase-3 cytoplasmic expression (p=0.330), and **C)** caspase-3 nuclear expression (p=0.412)

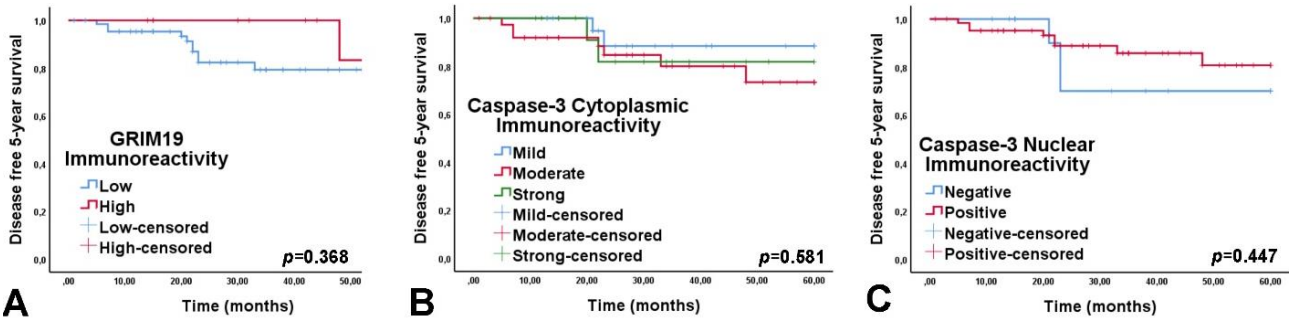

Figure 5. The Kaplan-Meier curves of GRIM-19 expression, caspase-3 cytoplasmic expression, and caspase-3 nuclear expression for 5-year disease-free survival. **A)** the 5-year DFS rate showed no association with GRIM-19 expression (p=0.368), **B)** caspase-3 cytoplasmic expression (p=0.581), and **C)** caspase-3 nuclear expression (p=0.447)

tumor histology (p=0.256), histologic grading (p=0.399), tumor stage (p=0.916), pT stage (p=0.264), lymphovascular invasion (p=0.139), lymph node metastases (p=0.534), perineural invasion (p=0.779), and presence of necrosis (p=0.384).

DISCUSSION

Invasive non-mucinous lung adenocarcinoma represents the most prevalent pathologic subtype among lung adenocarcinomas (2). The tumor growth rate depends on the balance between cellular proliferation and apoptosis, which determines tumor progression and prognosis (3). Caspase enzymes play an essential role in the apoptosis cascade. Besides their well-known apoptotic effects, caspase-3 may also cause spontaneous DNA double-strand breaks, chromosomal instability, and gene mutations, and promote malignant transformation through sublethal caspase-3 activation in cancer cells (6). Furthermore, caspase-3 has proliferative and angiogenic effects in tumor cells (4).

Caspase-3 overexpression has been demonstrated in many tumors (7). There is ongoing research on the significance of caspase-3 expression in the prognosis of malignant tumors, and the results are controversial. Several studies have shown that patients with breast, stomach, ovarian, cervical, and colorectal cancer, which exhibit high caspase-3 expression, have a worse prognosis (16,19). However, while some studies have indicated that a high level of caspase-3 expression is associated with better survival in patients with gastric and colorectal cancer (20,21), others have reported no significant relationship between caspase-3 expression and survival (22,23). Limited studies have been conducted on lung adenocarcinomas, and some of them have revealed that high caspase-3 expression is associated with longer survival rates in lung adenocarcinoma patients (24-26). However, Takata et al. (17) reported that patients with caspase-3 negative stage I lung adenocarcinoma had a higher 5-year OS rate compared to patients with caspase-3 positive tumors. In contrast to these results, our study demonstrated that caspase-3 positivity had no significant effect on 5-year OS or DFS rates.

Most of the available studies on lung carcinomas have not revealed any relationship between caspase-3 expression and prognostic factors such as histologic type, histologic grading, tumor stage, lymphovascular invasion, and lymph node involvement (17,24,25). However, Koomagi et al. (26) reported a significant association between caspase-3 expression and a lower incidence of lymph node involvement. In contrast, our study indicated that tumors with lymph node metastases and advanced tumors exhibited strong cytoplasmic expression of caspase-3, and there was a significant association between cytoplasmic caspase-3 expression and lymph node metastasis and tumor stage. Furthermore, we found that caspase-3 nuclear positivity was significantly associated with tumor histology and histologic grading in our current study. We observed that high-grade non-mucinous lung adenocarcinomas had a higher frequency of caspase-3 nuclear positivity. These findings suggest that nuclear

positivity and high cytoplasmic caspase-3 expression may be indicative of more aggressive behavior.

In addition to the well-known apoptosis pathways, rigorous research is ongoing to explore different mechanisms of cell death. GRIM-19, an apoptotic protein induced by the combination of IFN-β and RA, has emerged as a novel tumor suppressor (8,13). Overexpression of GRIM-19 has been shown to induce cell death through apoptosis (11,13,14). Notably, GRIM-19 expression is generally higher in normal tissues compared to malignant forms in various organs (27,28). Loss or reduced expression of GRIM-19 has also been observed in several tumors, including colorectal, breast, ovarian, cervical, and kidney tumors (7,28-31). Consistent with previous research, our study revealed that a majority of non-mucinous lung adenocarcinomas in our series (85.2%) exhibited low GRIM-19 expression. Furthermore, existing literature demonstrates a significant association between the loss or reduced expression of GRIM-19 and poor tumor differentiation, tumor stage, lymph node metastasis, and lymphovascular invasion in colorectal, breast, ovarian, and kidney tumors (28-31).

In the current literature, only a limited number of studies have investigated the impact of GRIM-19 on lung adenocarcinomas (15,27,32,33). Similar to our findings, Fan et al. (27) and Zhou et al. (32) reported a significant decrease in GRIM-19 expression in advanced lung adenocarcinomas compared to early-stage tumors. However, Wu et al. (33) did not observe a significant association between GRIM-19 expression and any histopathological findings, including tumor stage. In contrast to these studies, our research demonstrated that GRIM-19 expression was lower in tumors with higher histologic grades than in those with lower histologic grades. The loss of GRIM-19 expression exhibited a significant association with histologic grading. We also observed that tumors with lymphovascular invasion had lower levels of GRIM-19 expression compared to those without invasion.

Furthermore, our study revealed an interesting finding that patients with high GRIM-19 expression tumors had a higher 5-year OS rate compared to those with low GRIM-19 expression tumors, which has not been previously reported in the literature (92% and 48%, respectively). However, we did not observe a significant impact of GRIM-19 expression on the 5-year OS rate. It is worth noting that only a few studies in the available literature have investigated the significance of GRIM-19 expression on survival in malignant tumors. Hao et al. (29) reported that colon carcinoma patients with GRIM-19 negative tumors exhibited higher recurrence and metastatic rates, and negative tumors were associated with worse OS. Similarly, Ilelis et al. (18) demonstrated that patients with low GRIM-19 expression had worse OS and DFS rates compared to those with high GRIM-19 expression in high-grade serous ovarian carcinoma.

This study had several limitations. Firstly, the assessment of caspase-3 and GRIM-19 protein expressions were performed using TMA cores, which may have inherent limitations in terms of assessment accuracy. To mitigate this potential error, TMA blocks were obtained by sampling two cylindrical samples, each with a diameter of 2 mm, from different regions of each tumor. Secondly, protein expression was detected using immunohistochemistry, which may be subject to variability and subjective interpretation. Thirdly, the study was conducted at a single center, resulting in a limited number of patients included in our study. Therefore, it is crucial to validate these findings through additional multicenter studies involving a larger and more diverse patient cohort.

CONCLUSION

The expressions of caspase-3 and GRIM-19 may potentially contribute to tumorigenesis and tumor progression. Our study revealed significant associations between caspase-3 nuclear positivity, high cytoplasmic caspase-3 expression, and low GRIM-19 expression with unfavorable clinicopathological features, including tumor histology, tumor grade, tumor stage, lymphovascular invasion, and lymph node metastases. Moreover, our study demonstrated, for the first time in the literature, the significance of low GRIM-19 expression on the 5-year OS rate in patients with non-mucinous lung adenocarcinomas. These findings suggest that caspase-3 and GRIM-19 expression could serve as predictive markers for clinical behavior and potential targets for novel treatment options. However, further multicentric studies involving a larger number of patients are needed to determine the precise impact of these proteins in patients with non-mucinous lung adenocarcinoma.

Ethics Committee Approval: The study was approved by the Clinical Research Ethics Committee of Başkent University (27.04.2023, 162).

Conflict of Interest: None declared by the authors.

Financial Disclosure: None declared by the authors.

Acknowledgments: None declared by the authors.

Author Contributions: Idea/Concept: AOA, MT, EYA; Design: AOA, HÖ; Data Collection/Processing: AOA, EYA, LH; Analysis/Interpretation: AOA, MT, EYA; Literature Review: AOA, LH; Drafting/Writing: AOA, MT, EYA, HÖ; Critical Review: AOA, MT, EYA, DK, HÖ.

REFERENCES

1. WHO Classification of Tumours Editorial Board. Thoracic Tumours. 5th ed. Lyon, France: International Agency for Research on Cancer; 2021.
2. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2021. CA Cancer J Clin. 2021;71(1):7-33.
3. Haura EB, Cress WD, Chellappan S, Zheng Z, Bepler G. Antiapoptotic signaling pathways in non-small-cell lung cancer: biology and therapeutic strategies. Clin Lung Cancer. 2004;6(2):113-22.
4. Eskandari E, Eaves CJ. Paradoxical roles of caspase-3 in regulating cell survival, proliferation, and tumorigenesis. J Cell Biol. 2022;221(6):e202201159.
5. Boice A, Bouchier-Hayes L. Targeting apoptotic caspases in cancer. Biochim Biophys Acta Mol Cell Res. 2020;1867(6):118688.

6. Liu X, Li F, Huang Q, Zhang Z, Zhou L, Deng Y, et al. Self-inflicted DNA double-strand breaks sustain tumorigenicity and stemness of cancer cells. Cell Res. 2017;27(6):764-83.
7. Boudreau MW, Peh J, Hergenrother PJ. Procaspase-3 overexpression in cancer: a paradoxical observation with therapeutic potential. ACS Chem Biol. 2019;14(11):2335-48.
8. Kalvakolanu DV, Nallar SC, Kalakonda S. Cytokine-induced tumor suppressors: a GRIM story. Cytokine. 2010;52(1-2):128-42.
9. Sun P, Nallar SC, Raha A, Kalakonda S, Velalar CN, Reddy SP, et al. GRIM-19 and p16(INK4a) synergistically regulate cell cycle progression and E2F1-responsive gene expression. J Biol Chem. 2010;285(36):27545-52.
10. Nallar SC, Kalvakolanu DV. GRIM-19: A master regulator of cytokine induced tumor suppression, metastasis and energy metabolism. Cytokine Growth Factor Rev. 2017;33:1-18.
11. Lufei C, Ma J, Huang G, Zhang T, Novotny-Diermayr V, Ong CT, et al. GRIM-19, a death-regulatory gene product, suppresses Stat3 activity via functional interaction. EMBO J. 2003;22(6):1325-35.
12. Moreira S, Correia M, Soares P, Máximo V. GRIM-19 function in cancer development. Mitochondrion. 2011;11(5):693-9.
13. Angell JE, Lindner DJ, Shapiro PS, Hofmann ER, Kalvakolanu DV. Identification of GRIM-19, a novel cell death-regulatory gene induced by the interferon-beta and retinoic acid combination, using a genetic approach. J Biol Chem. 2000;275(43):33416-26.
14. Huang Y, Yang M, Yang H, Zeng Z. Upregulation of the GRIM-19 gene suppresses invasion and metastasis of human gastric cancer SGC-7901 cell line. Exp Cell Res. 2010;316(13):2061-70.
15. Wang T, Yan XB, Zhao JJ, Ye J, Jiang ZF, Wu DR, et al. Gene associated with retinoid-interferon-induced mortality-19 suppresses growth of lung adenocarcinoma tumor in vitro and in vivo. Lung Cancer. 2011;72(3):287-93.
16. Hu Q, Peng J, Liu W, He X, Cui L, Chen X, et al. Elevated cleaved caspase-3 is associated with shortened overall survival in several cancer types. Int J Clin Exp Pathol. 2014;7(8):5057-70.
17. Takata T, Tanaka F, Yamada T, Yanagihara K, Otake Y, Kawano Y, et al. Clinical significance of caspase-3 expression in pathologic-stage I, nonsmall-cell lung cancer. Int J Cancer. 2001;96(Suppl):54-60.
18. Ilelis F, do Amaral NS, Alves MR, da Costa AABA, Calsavara VF, Lordello L, et al. Prognostic value of GRIM-19, NF-κB and IKK2 in patients with high-grade serous ovarian cancer. Pathol Res Pract. 2018;214(2):187-94.
19. Liu X, Jiang S, Tian X, Jiang Y. Expression of cleaved caspase-3 predicts good chemotherapy response but poor survival for patients with advanced primary triple-negative breast cancer. Int J Clin Exp Pathol. 2018;11(9):4363-73.
20. Huang KH, Fang WL, Li AF, Liang PH, Wu CW, Shyr YM, et al. Caspase-3, a key apoptotic protein, as a prognostic marker in gastric cancer after curative surgery. Int J Surg. 2018;52:258-63.
21. Noble P, Vyas M, Al-Attar A, Durrant S, Scholefield J, Durrant L. High levels of cleaved caspase-3 in colorectal tumour stroma predict good survival. Br J Cancer. 2013;108(10):2097-105.
22. Nassar A, Lawson D, Cotsonis G, Cohen C. Survivin and caspase-3 expression in breast cancer: correlation with prognostic parameters, proliferation, angiogenesis, and outcome. Appl Immunohistochem Mol Morphol. 2008;16(2):113-20.
23. Vranic A. Caspase-3 and survivin expression in primary atypical and malignant meningiomas. ISRN Neurosci. 2013;2013:626290.
24. Huang YL, Zhang GH, Zhu Q, Wu X, Wu LG. Expression levels of caspase-3 and gasdermin E and their involvement in the occurrence and prognosis of lung cancer. Cancer Rep (Hoboken). 2022;5(9):e1561.
25. Yoo Jy, Kim CH, Song SH, Shim BY, Jeong YJ, Ahn MI, et al. Expression of caspase-3 and c-myc in non-small cell lung cancer. Cancer Res Treat. 2004;36(5):303-7.
26. Koomägi R, Volm M. Relationship between the expression of caspase-3 and the clinical outcome of patients with non-small cell lung cancer. Anticancer Res. 2000;20(1B):493-6.
27. Fan XY, Jiang ZF, Cai L, Liu RY. Expression and clinical significance of GRIM-19 in lung cancer. Med Oncol. 2012;29(5):3183-9.
28. Wang Y, Yan Y, Yang M, Yang Z. Expressions and clinical significances of STAT3 and Grim19 in epithelial ovarian cancer. 3 Biotech. 2020;10(6):246.
29. Hao M, Shu Z, Sun H, Sun R, Wang Y, Liu T, et al. GRIM-19 expression is a potent prognostic marker in colorectal cancer. Hum Pathol. 2015;46(12):1815-20.
30. Yan N, Feng X, Jiang S, Sun W, Sun MZ, Liu S. GRIM-19 deficiency promotes clear cell renal cell carcinoma progression and is associated with high TNM stage and Fuhrman grade. Oncol Lett. 2020;19(6):4115-21.
31. Zhou T, Chao L, Rong G, Wang C, Ma R, Wang X. Down-regulation of GRIM-19 is associated with STAT3 overexpression in breast carcinomas. Hum Pathol. 2013;44(9):1773-9.
32. Zhou AM, Zhao JJ, Ye J, Xiao WH, Kalvakolanu DV, Liu RY. Expression and clinical significance of GRIM-19 in non-small cell lung cancer. Ai Zheng. 2009;28(4):431-5. Chinese.
33. Wu HM, Jiang ZF, Fan XY, Wang T, Ke-Xu, Yan XB, et al. Reversed expression of GRIM-1 and GRP78 in human non-small cell lung cancer. Hum Pathol. 2014;45(9):1936-43.