

Prognostic Investigations of Expression Level of Two Genes FasL and Ki-67 as Independent Prognostic Markers of Human Retinoblastoma

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In this study, expression of FasL and Ki-67 messenger RNA (FasL and Ki-67 mRNA) in human retinoblastoma (HRB) was examined by the immunohistochemistry method and quantitative real-time PCR. Positive expression of Ki-67 in tumor cells was detected in 16 of 30 patients (53.33%), and only 9 (30%) of the tissues from patients with retinoblastoma showed positive staining for FasL. Our results revealed that FasL expression was significantly higher in tumor tissue with invasion compared with the noninvasion form ($p=0.033$). Ki-67 expression was markedly increased in tumor tissues with invasion compared with the noninvasion group ($p=0.04$), but no significant correlation was found between FasL expression and differentiation ($p>0.05$). In addition, Ki-67 expression was strongly linked to differentiation ($p<0.002$). Expression of these FasL was correlated with shorter overall survival of patients, but its expression was not significantly associated with overall survival ($p=0.15$). The impact of Ki-67 expression on survival in patients was also evaluated. Ki-67 expression level was not found to be significantly associated with shorter survival (Kaplan–Meier; $p=0.09$). Univariate analysis revealed that massive choroidal invasion was correlated with poor prognosis. Taken together, the data suggest that massive choroidal invasion is also an important indicator of poor prognosis for HRB.

Key words: Retinoblastoma (RB); Ki-67 and FasL; Analysis; Expression; Molecular

INTRODUCTION

Retinoblastoma (RB) is the most frequent primary intraocular malignancy in childhood, and delayed diagnosis is markedly correlated with a high mortality rate due to intracranial and systemic metastases at a later stage^{1–4}. The incidence is 1 in 17,000, typically presenting in the first 5 years of life. There is an urgent need to find the molecular mechanisms underlying RB progression in order to identify therapeutic strategies.

Fas ligand (CD95L) is a 40-kDa type II transmembrane protein that belongs to the tumor necrosis factor (TNF) family. Fas ligand is induced in activated T lymphocytes and NK cells, tumor cells, and cells of immune-privileged sites^{5–7}. FasL and Fas are key regulators of apoptosis, and extrinsic apoptosis is achieved through the FasL expression in cancer cells^{8,9}. Expression of FasL

has been previously reported in many types of human malignancies. Furthermore, FasL expression analysis was applied to evaluate its association with the staging and aggressiveness of carcinomas^{9,10}. Nevertheless, there are conflicting findings regarding the effects of FasL action. Aberrant expression of FasL may trigger profound inflammation resulting in rapid tissue rejection in organ transplantation models^{11–13}. Fas/FasL expression has been observed to cause tumor-protecting immunomodulation, with a direct impact on patient prognosis^{14,15}. Antigen Ki-67 is a nucleus protein that can signify the extent and percentage of proliferating cells in many types of malignancies, including carcinomas and sarcomas. Previous studies indicated the predictive value of Ki-67 expression in tumors regarding advantageous treatment strategy for breast carcinoma. In addition, the prognostic value

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of Ki-67 was revealed in various malignancies. In this study, we aimed to identify the pathological roles of FasL and Ki-67 in RB patients and the correlation of FasL and Ki-67 to clinical parameters.

MATERIAL AND METHODS

Patients and Samples

Thirty formalin-fixed tissue blocks of RB were selected from patients who underwent primary enucleation at a tertiary eye care referral center in Tehran between 2009 and 2012. All patients were enrolled after giving informed written consent. Clinicopathological patient characteristics are summarized in Table 1. Hematoxylin and eosin (H&E) slides were reviewed by two pathologists, and differences were resolved by consensus. Tumor staging was performed based on the American Joint Committee on Cancer (AJCC) staging criteria.

Quantitative Real-Time PCR

In brief, total RNA was purified from tumor tissues using the RNeasy Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 10 g of total RNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Germany). Transcripts were quantified by real-time PCR on a Rotor Gene 6000 Real-Time PCR (Qiagen) using the SYBR Green Real-Time PCR Master Mix. Each sample was examined in triplicate, and GAPDH was applied as an internal control. In addition, the relative expression of markers was calculated using the comparative cycle threshold (CT) method.

Immunohistochemical Analysis

Immunohistochemistry staining was performed using Avidin/Biotin Complex (ABC) Kits. The tissue sections

Table 1. Correlation Between Clinicopathological Features and FasL/Ki-67 Expressions in Retinoblastoma Patients

	FasL			Ki-67		
	Negative (n=21)	Positive (n=9)	p Value	Negative (n=14)	Positive (n=16)	p Value
Age			NS			NS
<2 years	8	5		6	7	
≥2 years	13	4		8	9	
Gender			NS			NS
Male	11	6		10	7	
Female	10	3		4	9	
Laterality			NS			NS
Unilateral	7	4		6	5	
Bilateral	14	5		8	11	
Differentiation of tumor			NS			0.002
PDRB	12	3		4	11	
WDRB	9	6		10	5	
Staging						
T1N0M0	11	4	NS	8	7	NS
T2aN0M0–T4bN0M0	10	5		6	9	
Invasion of choroid						
Massive	4	8	0.001	2	10	0.001
Focal	17	1		12	6	
Invasion of sclera			NS			
Yes	11	5		7	9	NS
No	10	4		7	7	
Invasion of iris and ciliary body			NS			
Yes	7	6		5	8	NS
No	14	3		9	8	
ON invasion (RL and cut end)			NS			
Yes	9	7		6	10	NS
No	12	2		8	6	
Tumor invasion			0.033			0.04
Yes	3	8		4	7	
No	18	1		17	2	

NS, not significant.

were deparaffinized in xylene, rehydrated in graded ethanol solutions, and then antigen retrieval was performed using citrate buffer (pH 6.0) for 20 min, followed by incubation of sections with 3% hydrogen peroxide in methanol for 30 min. Then the sections were incubated with Rabbit FasL Polyclonal Antibody [FasL (P137); 1:50; Bioworld Technology Inc., Minneapolis, MN, USA] and Rabbit Polyclonal to Ki-67 (ab15580; Abcam, Cambridge, MA, USA). Afterward, the slides were stained using the labeled streptavidin–biotin II method. Visualization was developed using 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) as chromogen and counterstained with hematoxylin. Nonneoplastic tissues were applied as control tissues, and nonimmune IgG was also used as a negative control antibody.

Evaluation of Staining

The results were evaluated according to a semiquantitative grading system based on both the proportion of stained cells and their intensity. In this study, about 2,000 tumor cells from 20 systematically randomized fields (400×) were counted, and the percentage of positive cells was calculated. The percentage scoring of immunoreactive tumor cells was as follows: 0, <1 positive tumor cells; 1, 1%–10%; 2, 11%–50%; 3, 50%–75%; 4, >75% positive tumor cells. Intensity was evaluated in comparison with the control and scored as follows: negative staining (0), weak staining (1+), moderate staining (2+), and strong staining (3+). Then we added the scores of intensity and percentage as a final score ranging from 0 to 7. The results of immunostaining were divided into two groups, 0–2 was counted as negative (–), and 3–7 was considered positive (+).

Western Blot Assay

Western blot analysis was applied to detect proteins using standard methods. The tissue was homogenized on ice in PBS and lysed in RIPA buffer. Lysates were cleared by centrifugation (14,000 rpm) at 4°C for 10 min. Then the supernatant was diluted in 2× SDS loading buffer and denatured at 100°C for 10 min. Protein samples were electrophoresed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) filter membrane. The primary anti-FasL antibody (Bioworld Technology Inc.), anti-Ki-67 antibody (Abcam), and anti-GAPDH antibody (Abcam) were diluted to 1:500 and incubated with membrane overnight at 4°C.

Statistical Analysis

All computations were performed using the SPSS 19.0 software for the analysis of all data. The statistical correlations between FasL and Ki-67 and the clinicopathological factors were analyzed using the Fisher's exact

test. The Kaplan–Meier method and log-rank test were utilized for survival analysis. In addition, multivariate Cox regression analysis was performed using the stepwise Cox regression model to assess the effect of multiple independent prognostic factors on survival outcome. The significance level was set at $p < 0.05$. All values were expressed as mean ± SEM (standard error of the mean).

RESULTS

Statistical Association of Protein Expression With Clinicopathological Characteristics

Demographic characteristics and clinicopathological parameters are shown in Table 1. A total of 18 males and 12 females were included in the study, with median age of 3 years (SD=3.26, range=2 months–0 years). Among a total of 30 patients, the tumor tissues were categorized into two groups based on aggressiveness, including tumors with no invasion and tumors with invasion (the choroidal/scleral/iris and ciliary body/retrolaminar and optic nerve cut end invasion). Ki-67 mRNA expression was detected in 18 patients (60%) normalized to GAPDH, whereas only 12/30 (40%) tumor tissues showed mRNA expression levels of FasL (Fig. 1). Ki-67 and FasL protein expression levels were detected by immunohistochemistry assay in tumor tissues.

Positive expression of Ki-67 in tumor cells was observed in 16 of 30 patients (53.33%), while 46.66% of patients had negative expression. Moreover, only nine (30%) of the tissues from patients with RB showed positive staining for FasL (Fig. 2). FasL positivity was confined to the cytoplasm and membrane cells, and Ki-67 was mainly expressed in the nuclei.

Our results suggested that expression of FasL was significantly more common in tumor tissue with invasion than those with the noninvasion form ($p = 0.033$). Furthermore, Ki-67 expression was significantly elevated in tumor tissues with invasion compared with the noninvasion group ($p = 0.04$). Histopathological evaluation showed that 17 tumors were poorly differentiated RBs and that 11 cases showed the presence of necrosis, but no significant association was found between FasL expression and differentiation ($p > 0.05$) (Table 1). Ki-67 expression was significantly correlated with differentiation ($p < 0.002$) (Table 1). However, there was no obvious relevance to other clinicopathological parameters.

Expression of Proteins With Patient Outcome

Kaplan–Meier analysis and log-rank test showed that overall survival was lower in patients who had expression of FasL, but its expression was not significantly associated with overall survival ($p = 0.15$) (Fig. 3). Ki-67 expression level was not found to be significantly linked to shorter survival (Kaplan–Meier; $p = 0.09$) (Fig. 4).

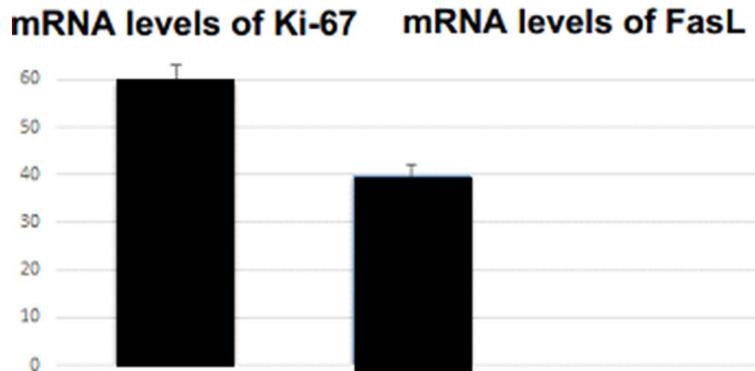


Figure 1. The expression level of mRNAs in patients with retinoblastoma (RB).

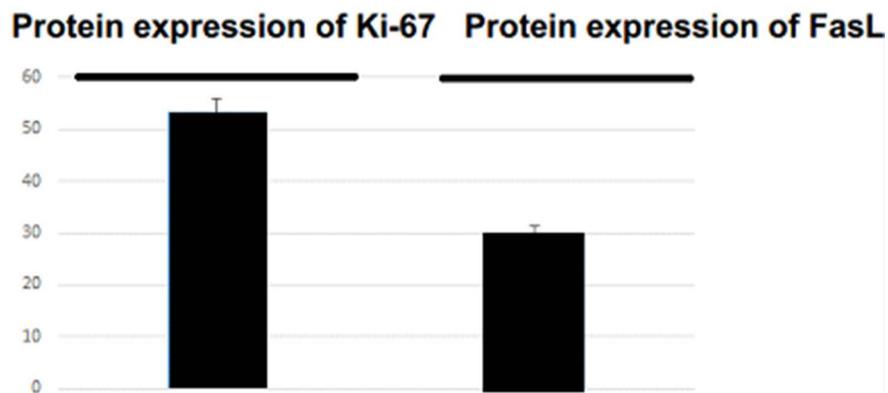


Figure 2. The expression level of proteins in patients with RB.

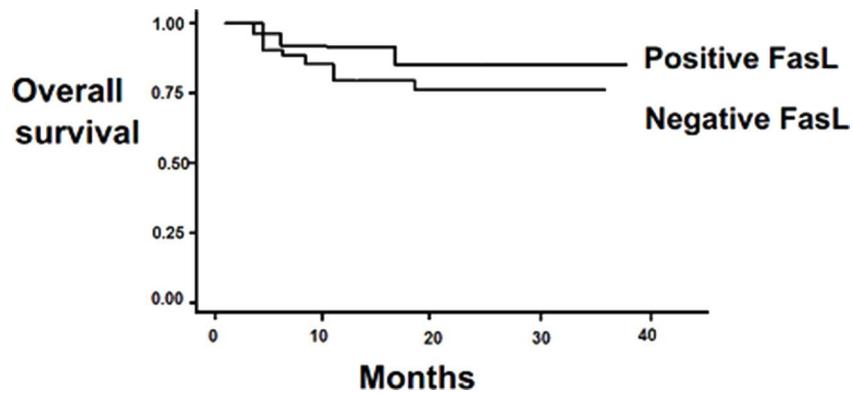


Figure 3. Correlation between FasL expression and overall survival in patients (Kaplan–Meier; $p=0.15$).

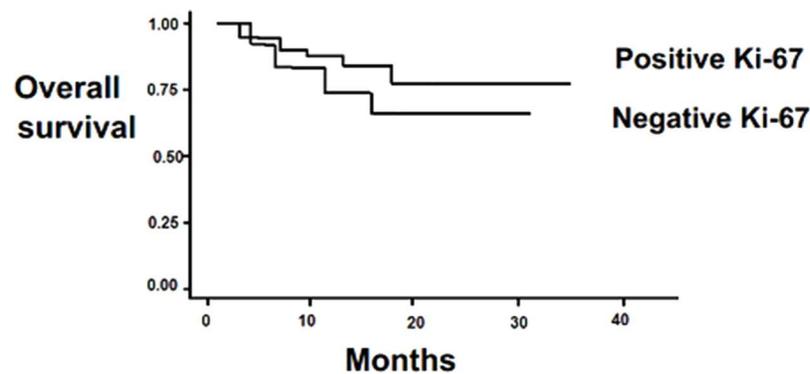


Figure 4. Correlation between Ki-67 expression and overall survival in patients (Kaplan–Meier; $p=0.09$).

Association of Clinicopathological Parameters of HRB by Univariate and Multivariate Analysis

Univariate Cox regression analysis also identified that massive choroidal invasion was related to poor prognosis (Table 2). Furthermore, multivariate Cox regression analysis suggested that massive choroidal invasion was also the important indicator of poor prognosis for RB (Table 2).

Detection of FasL and Ki-67 in Tissue Samples by Western Blot

Western blot analysis was performed in order to measure the expression of FasL and Ki-67 protein within the RB tissues. The results revealed that FasL and Ki-67 protein expression was significantly higher in the RB tissues than in the control group tissues (Fig. 5).

Taken together, these results suggest that FasL and Ki-67 protein expression is correlated with the invasion of RB.

DISCUSSION

Aberrant expression of Fas and/or FasL has been reported in many kinds of malignancies^{9,10}. Decreased

Fas and increased FasL expression have been observed in many types of cancers, such as bladder cancer. High expression of FasL has been reported to increase the ability of tumor cells to counterattack the immune system by inducing apoptosis of Fas-sensitive lymphocytes. On the other hand, low expression of Fas may be correlated with protection of tumor cells from elimination via antitumor immune responses¹². Furthermore, aberrant expression of FasL may trigger profound inflammation, resulting in rapid tissue rejection in organ transplantation models^{11,13}. The Fas–FasL system has been shown to be one of the main signaling pathways in apoptotic cell death.

Results showed the majority of our cases had mRNA levels of Ki-67 [in 18/30 (60%)], whereas only 12/30 (40%) of the tumor tissues showed mRNA levels of FasL. In the present study, protein expression of FasL was shown in nine cases (30%). FasL has been shown to be expressed in melanomas, colon cancer, choriocarcinoma, and breast cancer. Studies indicated that cancer cells induce apoptosis in Fas-sensitive, but not in Fas-insensitive, lymphoma cells¹⁵. Studies suggested that FasL-induced suppression of tumor-specific Fas-bearing T cells may be associated with neoplastic cells that escape from immune surveillance.

Table 2. Univariate and Multivariate Analysis of Prognostic Parameters by Cox

Clinicopathological Characteristics	Relative Risk (RR)	Univariate Log-Rank Test (p)	Cox Multivariable Analysis (p)
Age	0.554	0.476	0.631
Sex	0.923	0.674	0.81
Laterality	0.773	0.616	0.551
Differentiation of tumor	1.23	0.273	0.413
Staging	0.823	0.345	0.532
Invasion of choroid (massive)	2.341	0.003	0.029
Invasion of sclera	0.745	0.452	0.623
Invasion of iris and ciliary body	0.689	0.534	0.632
ON invasion (RL and cut end)	0.932	0.453	0.562
FasL expression	1.15	0.378	0.423
Ki-67	1.45	0.278	0.364

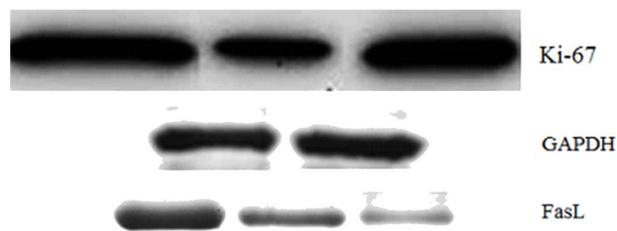


Figure 5. Western blot analysis of proteins in tissues.

Saigusa et al.¹⁶ reported that patients with high FasL expression had poorer recurrence-free and overall survival in esophageal squamous cell carcinoma. In contrast, our results suggest that overall survival was lower in patients who had expression of FasL, although this did not reach any statistical significance (Kaplan–Meier; $p=0.23$). In agreement with a study by Saggiaro et al.¹⁷, we did not find any association between FasL expression and survival. Furthermore, aberrant expression of FasL may trigger profound inflammation, resulting in rapid tissue rejection in organ transplantation models^{11,13}. The Fas–FasL system has been shown to be one of the main signaling pathways for apoptotic cell death^{18,19}.

It was determined that FasL expression was correlated with poor prognosis in both tumor cells and the tumor-associated vessels. The important role of FasL in tumorigenesis has been shown in colon cancer²⁰. Other investigations have shown that higher FasL expression is likely to lead to a worse prognosis in esophageal and lung tumors^{21–24}.

There are conflicting findings regarding the expression of FasL in RB. Krishnakumar²⁵ has indicated that FasL is expressed in RB specimens from tumors with invasion of the choroid (diffuse invasion), optic nerve, and orbit, while FasL is negative in tumor tissues with no invasion. Krishnakumar indicated that increased expression of FasL is associated with aggressive RB. In accordance with the study conducted by Krishnakumar, our results suggested that expression of FasL is increased in tumor tissue with invasion when compared with the noninvasion group, but our findings did not find an association between the FasL expression level and differentiation. Loss of Fas and gain of ectopic FasL expression are common in malignant transformation. It has been reported that the Fas/FasL pathway is immunosuppressive and may be associated with the escape of HRB cells from immune destruction²⁵. Despite the current evidence, the factors regulating FasL expression on cancer cells, including those in RB, need further investigation.

Ki-67 expression has been reported to be strongly associated with the growth fraction in several model systems, and its expression has been previously evaluated in many types of human malignancies²⁶. It has been

indicated that Ki-67 proliferative activity is linked to the extent of tumor differentiation, invasion, metastasis, and prognosis²⁷.

In the present study, Ki-67 mRNA expression was increased in tumor tissues (60%). Our result is achieved in accordance with Yuan et al.²⁸, who found increased Ki-67 mRNA expression in breast cancer. They indicated that Ki-67 is strongly linked to the pathogenesis and development of breast cancer.

In the present study, Ki-67 protein expression levels were also detected by immunohistochemistry assay in tumor tissues. Positive expression of Ki-67 in tumor cells was observed in 53.33% of patients. It has been suggested that Ki-67 expression was increased in prostate cancer^{29,30}. Inwald et al.³¹ have reported a strongly increased Ki-67 expression in breast cancer. Those findings corroborate the findings of our present study.

Furthermore, Ki-67 expression was significantly elevated in tumor tissues with invasion compared with the noninvasion group ($p=0.04$). The results revealed that the level of Ki-67 expression was significantly correlated with differentiation, which is in accordance with other investigations reporting on the association of Ki-67 gene expression with clinicopathological factors of other malignancies^{32,33}. However, there was no obvious relevance to other clinical parameters in our study. Ki-67 expression in breast cancer tissue has been reported to be positively associated with the TNM stages, the tumor size, and the number of metastatic lymph nodes. These results showed that increased Ki-67 expression is involved in the promotion of the pathogenesis and development of breast cancer²⁸. Zheng et al.³⁴ demonstrated that the knockdown of Ki-67 inhibits cancer cell proliferation and promotes cell apoptosis, and Ki-67 may be implicated in the progression of renal carcinoma. The increased expression of Ki-67 suggested the aggressiveness of tumors that are characterized by an increase in the number of cells undergoing mitosis³⁵. The prognostic value of Ki-67 for survival may be different in various tumor tissues.

In this study, Ki-67 expression was related to poorer survival of patients ($p=0.12$). The Ki-67 index has been revealed to be associated with tumor angiogenesis and the survival rate in patients with multiple myeloma³⁶. Furthermore, Ki-67 expression has been found to be linked to survival in renal cell carcinoma³⁷. It has been indicated that high Ki-67 expression may indicate better survival than low Ki-67 expression in muscle-invasive bladder cancer (MIBC) patients treated with chemoradiotherapy³⁸.

Several studies have indicated that high Ki-67 expression in a tumor is a prognostic factor in NSCLC^{39,40}. Similar to our findings, investigations published in 2004 suggested that increased expression of Ki-67 was correlated with a shorter overall survival in NSCLC⁴¹.

Ki-67 is considered to be a protein correlated with cell cycle activity and growth fraction, and its prognostic and predictive values were revealed in various types of malignancies such as breast cancer. However, further investigation is needed to identify the mechanisms involved in RB progression for therapeutic strategies. Multivariate analysis suggested that massive choroidal invasion can be an important prognostic indicator of RB.

In summary, higher Ki-67 and FasL expressions were significantly associated with invasion in tumor tissues. We speculate that massive choroidal invasion can be an important prognostic indicator of HRB.

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