Int J Clin Exp Pathol 2020;13(9):2312-2318
www.ijcep.com /ISSN:1936-2625/IJCEP0114623

Original Article

Kank1 and Ki67 expression are associated with poor prognosis in human pulmonary adenocarcinoma

Xiao-Chun Shen[1*], Chong-Jun Ni[1*], Song-Tao Xu[3,4], Sheng-Hua Zhan[2], Guo-Jian Gu[5,6]

Departments of [1]Respiratory Medicine, [2]Pathology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, PR China; [3]Department of Clinical, Luohe Medical College, Luohe, Henan, PR China; [4]Tumor Occurrence and Prevention Research Innovation Team of Henan, Luohe, Henan, PR China; [5]Department of Pathology, Taicang Affiliated Hospital of Soochow University, Taicang, Jiangsu, PR China; [6]Department of Pathology, The First People's Hospital of Taicang, Taicang, Jiangsu, PR China. [*]Equal contributors.

Received May 19, 2020; Accepted June 29, 2020; Epub September 1, 2020; Published September 15, 2020

Abstract: KN motif and ankyrin repeat domains 1 (Kank1) and ki67 are associated with tumorigenesis and progression. This paper researched the expression of Kank1 and Ki67 and their clinicopathologic significance in pulmonary adenocarcinoma (PA). We monitored the expression of KanK1 and ki67 in 94 cases of human PA and 31 cases of paracancerous tissue by the immunohistochemical method. The results showed that Kank1 protein was detected in 74.2% (41/94) of PA tissues, and they were associated with differentiation (P = 0.025) and lymphatic metastasis (P = 0.002). Kaplan-Meier analysis suggested that patients with low Kank1 expression had shorter overall survival in PA (P = 0.020). Ki67 protein was detected in 79.8% (75/94) of PA tissues, and they were associated with differentiation (P < 0.001), TNM classification (P = 0.007), and lymphatic metastasis (P = 0.044). Furthermore, Kaplan-Meier analysis showed that patients with overexpression of Ki67 had shorter overall survival (P = 0.014). Cox multivariate analysis showed that tumor differentiation, TNM classification, lymphatic metastasis, Kank1, and ki67 expression were independent factors for prognosis of PA (P = 0.012, 0.016, 0.007, 0.021 and P = 0.003 respectively). In conclusion, compared with paracancerous tissues, Kank1 had low expression, while Ki67 was overexpressed in PA. They are closely related to its occurrence and development, and the prognosis of patients with low expression of Kank1 or overexpression of ki67 was poor in PA. Kank1 and Ki67 can be helpful for diagnosing and detecting the prognosis of patients with PA.

Keywords: Kank1, ki67, immunohistochemistry, pulmonary adenocarcinoma, prognosis

Introduction

Lung cancer is one of the most common malignant tumors in the respiratory system and is the main cause of cancer-related death in China [1]. The incidence of adenocarcinoma is increasing year by year, and itis also the most prevalent subtype. The high death rate of this cancer is due to the fact that many patients have been diagnosed with advanced stage of cancer, which has metastasized so they lost the opportunity of operation [3, 4]. Only palliative treatment can be chosen. According to the study, the 5-year survival rate of patients with its advanced stages is only 10-15% [5]. Therefore, we are in need of finding new and convenient tumor detection indicators, improving the detection of early lung cancer in the curable stage, thus reducing the death rate of patients, and prolonging their lives.

In recent years, the role of KANK1 in the occurrence and development of malignant tumors has claimed the attention of many scholars. Kank1 gene is a member of the kank family, also known as ANKRD15, which is located on chromosome 9p24 [6-8].

It contains the central coiled-coil domains, N-terminal KN motif, and the C-terminal ankyrin (ANK) repeat domains [8, 9]. It is a new candidate tumor suppressor gene found in renal cell carcinoma [9, 10]. The deletion or mutation of tumor suppressor gene is one of the factors leading to abnormal gene expression and functional inactivation. Kank1 has been reported to

be absent in many tumor diseases, such as human breast cancer, nasopharyngeal carcinoma, oral squamous cell carcinoma, and gastric cancer cells [9-12].

Ki67 is an important tumor proliferation marker, which is overexpressed in the cell proliferation cycle (G1, S, G2 and M), but has rare expression in resting G0 cells [13, 14]. Prognosis in patients with high expression of Ki67 was worse than that of patients with low expression [15-17].

Kank1 protein is rarely studied in lung cancer, and its specific mechanism is still unknown. In this study, the expression of Kank1 and Ki67 were detected by immunohistochemistry in lung cancer tissue, and the relationship between them and their clinicopathological features were analyzed, so as to provide the basis for the discovery of markers conducive to the diagnosis of lung cancer.

Materials and methods

Materials

From October 2014 to October 2019, 94 paraffin specimens of lung cancer and 31 paracancerous tissue specimens were surgically removed in the First Affiliated Hospital of Soochow University. They were confirmed to be pulmonary adenocarcinoma by two pathologists. The average age of the patients was 64.9 years old, ranging from 48-83 years old. TNM classification system was implemented by American Joint Committee on Cancer (AJCC) in 2010, and all patients with PA did not receive chemotherapy or radiotherapy before operation [18]. All patients with lung adenocarcinoma were followed by telephone for 3-60 months.

Immunohistochemical analysis

This was similar to previous papers [19]. In short, all specimens were sliced and then baked at 68°C for 20 minutes. Specimens were dewaxed with xylene, and dehydrated with alcohol. They were treated with 3% H_2O_2 for 10 minutes, and washed with phosphate buffer saline (PBS) 3 times for 15 min. Then they were soaked in citric acid buffer and boil (95°C, 15-20 min). Sheep serum working solution was used to block antigens and incubated at 37°C for 10 min. Then Rabbit anti human Kank1 antibody (1:150, Santa Cruz Biotechnology, Inc; Santa Cruz, CA, USA) or Ki67 antibody (1:100, Maixin Biotechnology Co. Ltd; Fuzhou, China) was used to incubate in refrigerator at 4°C overnight and washed with PBS 3 times for 15 min (PBS buffer was used to replace the first antibody as negative control). Then we added the secondary antibody. The samples were incubated with streptavidin horseradish peroxidase, incubated at 37°C for 30 min. DAB was used for visualization, and washed with water fully, then stained with hematoxylin again. Specimens were washed to colorless.

Evaluation of Kank1 and Ki67 staining

This was the classification standard of staining intensity: 0 (no staining), 1 (light staining), 2 (moderate staining), 3 (heavy staining). This was the grading standard for the staining area: 0 (No staining), 1 (< 30% tumor cells were stained), 2 (30~60% tumor cells were stained), 3 (> 60% tumor cells were stained). The expression of kank1 and Ki67 was evaluated by the product of the proportional score and intensity score. When the score was less than 3, the expression was defined as negative (-, low expression) and when the score was 3-6, the expression was defined as positive (+, overexpression) [11].

Statistical analysis

Version 16.0 of SPSS was used to statistical analysis. Classified variables were analysis by Chi square test. The survival data were determined by Kaplan Meier method. Graphpad prism version 6.01 was used for survival curves. Cox proportional hazard model was used to multivariate analysis. When $P < 0.05$, a difference was considered significant.

Results

Expression of Kank1 and Ki67

Kank1 had mainly cytoplasmic staining, with yellow granules in the cytoplasm of PA (**Figure 1A**). Ki67 had mainly nuclear staining, with yellow granules in the nucleus (**Figure 1C**). Positive expression rate of Kank1 was 43.6% in PA, which was clearly lower than that in paracancerous tissues (56.4%), $P = 0.003$, (**Table 1**). Negative expression of Kank1 was obviously relevant to PA lymphatic metastasis and differentiation ($P = 0.002$ and $P = 0.025$, respective-

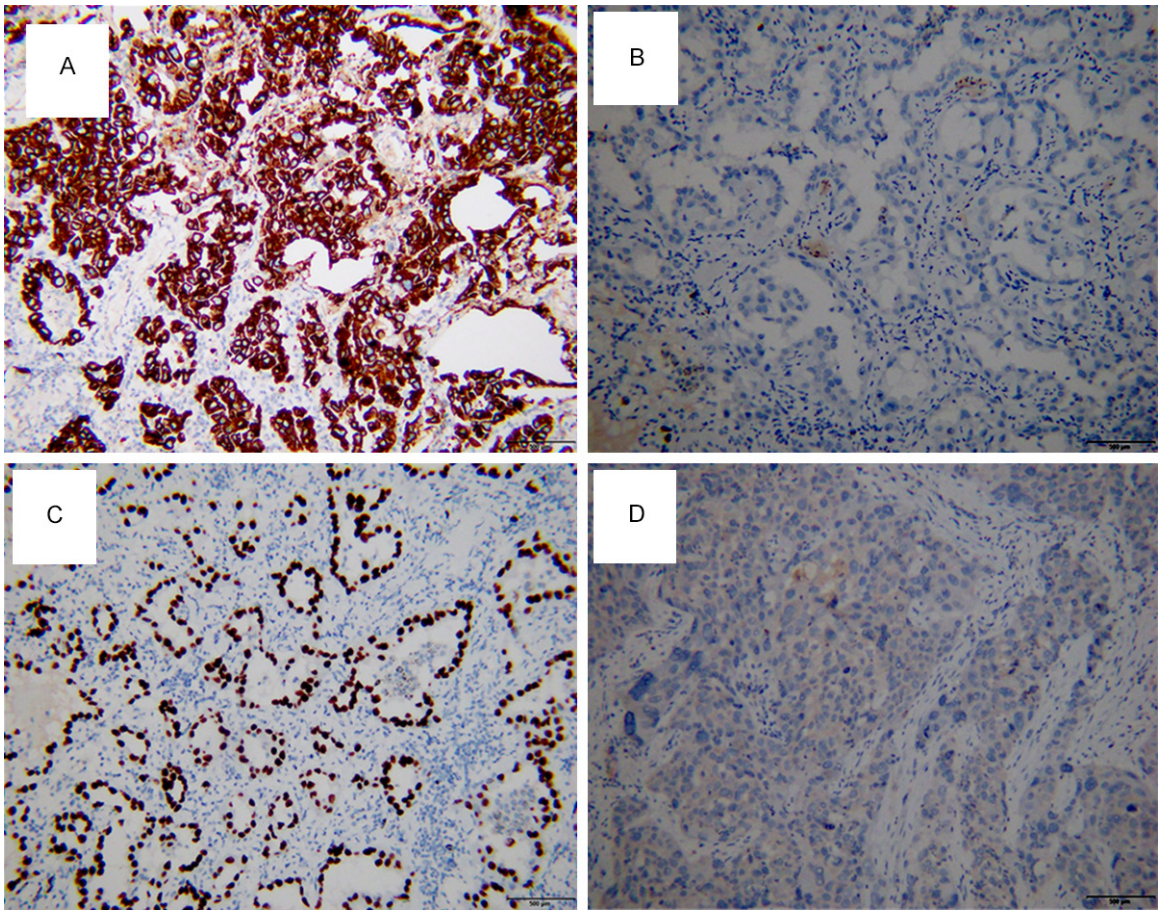

Figure 1. Kank1 had positive expression in pulmonary adenocarcinoma tissues manifested by brown granules in the cytoplasm (200×) (A). Kank1 was not expressed in pulmonary adenocarcinoma tissues and the cells were not stained (200×) (B). Ki67 had positive expression in pulmonary adenocarcinoma tissues and there were brown granules in the nuclei (200×) (C). Ki67 was not expressed in PA (200×) (D).

ly). However, Kank1 expression had a link with sex, tumor size, age, and TNM classification (P = 0.628, P = 0.147, P = 0.543 and P = 0.076, respectively), as shown in **Table 2**. The positive expression rate of Ki67 was 79.8% in human PA that was clearly higher than that in paracancerous tissues (16.1%), P < 0.001, (**Table 1**). The positive expression of Ki67 was relevant to PA differentiation, TNM classification, and lymphatic metastasis (P < 0.001, P = 0.007 and P = 0.044, respectively). However, Ki67 expression was not correlated with sex, tumor size, and age (P = 0.424, P = 0.867 and P = 0.0573, respectively), as was shown in **Table 2**.

Expression of Kank1 and ki67 correlate with survival

Kank1 negative patients' overall survival rate was significantly lower than that of positive patients (P = 0.020, log-rank test), (**Figure 2A**). The overall survival rate of patients with positive Ki67 expression was significantly lower than that of patients with negative Ki67 expression (P = 0.014, log-rank test), as was shown in **Figure 2B**). Cox multivariate analysis suggested that PA differentiation, TNM classification, and lymphatic metastasis, Kank1, and Ki67 expression were independent factors for prognosis (P = 0.012, P = 0.016, P = 0.007, P = 0.021 and P = 0.003 respectively), as shown in **Table 3**.

Correlations between Kank1 and ki67 expression

The expression of Kank1 was negatively correlated with that of ki67 in PA (r = -0.465, P < 0.001), **Table 4**.

Discussion

Previous studies had shown that Kank1 expression was absent or downregulated in a variety

Table 1. Expression of Kank1[a] and Ki67 in PA and paracancerous tissue

Related Factor	Pulmonary adenocarcinoma tissue		paracancerous tissue		*P* Value
	Negative (-)	Positive (+)	Negative (-)	Positive (+)	
Kank1	53 (56.4)	41 (43.6)	8 (25.8)	23 (74.2)	0.003
Ki67	19 (20.2)	75 (79.8)	26 (83.9)	5 (16.1)	< 0.001

[a]KN motif and ankyrin repeat domains 1.

Table 2. Analysis of Kank1[a] and Ki67 positive expression and related factors

Related Factor	n	Kank1 expression		*P* Value	Ki67 expression		*P* Value
		Negative (-)	Positive (+)		Negative (-)	Positive (+)	
Sex							
Male	37	22 (59.5)	15 (40.5)	0.628	9 (24.3)	28 (75.7)	0.424
Female	57	31 (54.4)	26 (45.6)		10 (17.5)	47 (82.5)	
Tumor size (cm)							
< 3	38	18 (47.4)	20 (52.6)	0.147	8 (21.1)	30 (78.9)	0.867
≥ 3	56	35 (62.5)	21 (37.5)		11 (19.6)	45 (80.4)	
Age (years)							
≥ 60	54	29 (53.7)	25 (46.3)	0.543	12 (22.2)	42 (77.8)	0.573
< 60	40	24 (60.0)	16 (40.0)		7 (17.5)	33 (82.5)	
Differentiation							
Well	32	13 (40.6)	19 (59.4)	0.025	15 (46.9)	17 (53.1)	< 0.001
Poor + moderate	62	40 (64.5)	22 (35.5)		4 (6.5)	58 (93.5)	
TNM classification							
I-II	43	20 (46.5)	23 (53.5)	0.076	14 (32.6)	29 (67.4)	0.007
III-IV	51	33 (64.7)	18 (35.3)		5 (9.8)	46 (90.2)	
Lymphatic metastasis							
yes	49	35 (71.4)	14 (28.6)	0.002	6 (12.2)	43 (87.8)	0.044
no	45	18 (40.0)	27 (60.0)		13 (28.9)	32 (71.1)	

[a]KN motif and ankyrin repeat domains 1.

of tumor types [9-12]. It contributes to the occurrence and development of tumor by several ways. First, by interacting with 14-3-3 protein, a downstream molecule of PI3K-Akt signaling pathway, Kank1 could inhibit the activation of RhoA, thus inhibiting actin polymerization and cell migration [20, 21]. Second, the apoptosis of human glioma cells was induced by the Kank1 gene through mitochondrial pathway, and the cell cycle was blocked in the G0/G1 phase [22]. Third, Fan's study found that Kank1 could inhibit the proliferation of tumor cells by regulating Yap to induce apoptosis [11]. In addition, Kank1 induces apoptosis of human peripheral nerve sheath malignant tumor cells by regulating CXXC5 [23].

In the study, compared with paracancerous tissue, the positive rate of Kank1 was significantly lower in PA tissue (P = 0.003). The worse the degree of PA differentiation, the lower of Kank1 expression. Compared with the group without lymphatic metastasis, its positive rate of lymphatic metastasis group was significantly lower. It suggested that the absence of Kank1 had an effect on the tumor occurrence and development, and could be used for the auxiliary diagnosis of PA.

The overall survival time of Kank1 negative patients was significantly longer than that of positive patients [9, 11]. Cox multivariate analysis indicated that its negative expression was an independent risk factor for PA prognosis. Our study also suggested that the prognosis of patients who were negative for it was poor. These results indicated that Kank1 protein was a good prognostic marker for PA.

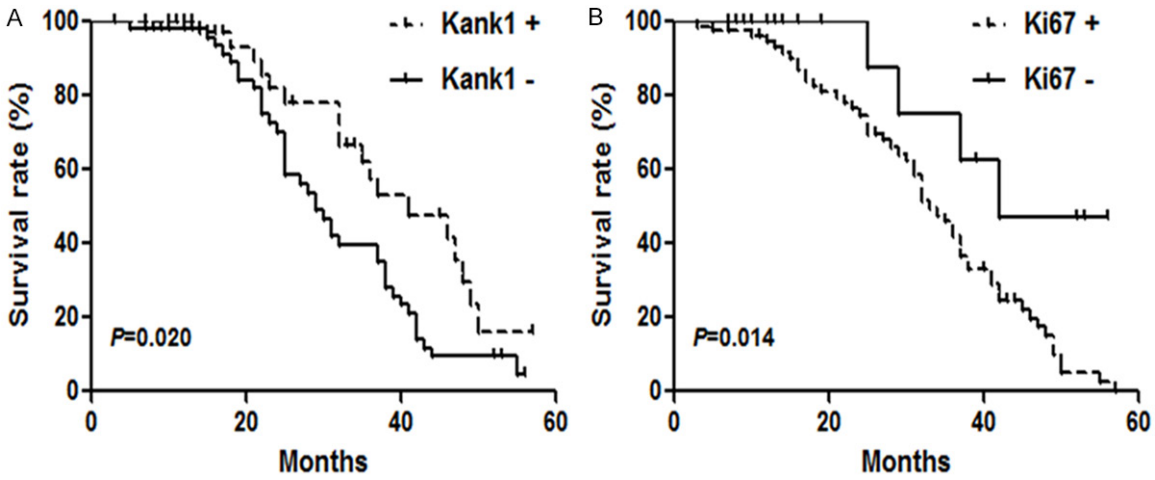

Figure 2. Kaplan-Meier survival curves of pulmonary adenocarcinoma patients according to Kank1 expression (A) or Ki67 expression (B).

Table 3. Cox analyses of different clinicopathologic variables and Kank1[a] and Ki67 expression in pulmonary adenocarcinoma tissues

Variable	Hazard Ratio	95% Hazard Ratio Confidence Limits		*p* value
Sex (Male vs. Female)	1.056	0.565	2.217	0.735
Tumor size (< 3 vs. ≥ 3)	1.852	0.763	3.241	0.196
Age (≥ 65 vs. < 65)	1.028	0.627	2.194	0.861
Differentiation (well vs. poor + moderate)	2.458	1.143	6.325	0.012
TNM classification (I-II vs. III-IV)	4.175	1.668	11.341	0.016
Lymphatic metastasis (no vs. yes)	3.856	1.645	7.359	0.007
Kank1 (positive vs. negative)	0.274	0.063	0.874	0.021
Ki67 (positive vs. negative)	4.126	1.352	11.419	0.003

[a]KN motif and ankyrin repeat domains 1.

Table 4. Correlations between Kank1[a] and Ki67 expression in pulmonary adenocarcinoma tissues

Ki67	Kank1		Contingency coefficien (r)	χ^2	P
	+	-			
+	24	51	-0.465	20.362	< 0.001
-	17	2			

[a]KN motif and ankyrin repeat domains 1.

Many scholars have found that up-regulation of Kank1 gene can inhibit the proliferation of tumor cells. With down-regulation of its expression, the result is opposite [11, 22]. This suggested that it might be a target for PA gene therapy.

Ki67 is up-regulated in tumors, and the overall survival time of patients with positive expression of Ki67 is shorter than that of patients with negative expression [15-17].

Compared with paracancerous tissues, the positive rate of Ki67 was much higher in PA tissue ($P < 0.001$). Compared with the group without lymphatic metastasis, its positive rate of lymphatic metastasis group was also (significantly) higher. It correlated with the stage by TNM classification. The worse the degree of PA differentiation, the higher of the Kank1 expression. This suggested that the ki67 was associated with tumor occurrence and development, and could be used for the auxiliary diagnosis of

PA. Our follow-up results also showed that patients who had positive expression had a poor prognosis. Cox multivariate analysis also suggested that its positive expression was an independent risk factor for prognosis of PA. It also showed that Kank1 was down regulated while Ki67 was up regulated, and they were inversely correlated in PA. This is similar to Fan's study that Kank1 had a regulatory effect on Ki67 [11].

However, there are some limitations in this study. The sample size was too small, and the research results may be biased. In the future, we will expand the sample size, conduct multi-center research, and further clarify the correlation.

Conclusion

Compared with the paracancerous tissues, Kank1 had low expression, while Ki67 was overexpressed in PA. They were closely related to its occurrence and development. The prognosis of patients with low expression of Kank1 or overexpression of ki67 was poor in PA. Kank1 and Ki67 can be used to assist in the diagnosis and detect the prognosis of patients with PA.

Acknowledgements

The research was supported by (a) innovation, entrepreneurship and development ability improvement of engineering and scientific research projects in 2020 at Luohe Medical College in Henan Province P.R.C. (2020-LYZK-YZD006), (b) the Science and Technology Development Plan of Taicang in Jiangsu Province P.R.C. (No. TC2017SFYL01).

Disclosure of conflict of interest

None.

Address correspondence to: Guo-Jian Gu, Department of Pathology, Taicang Affiliated Hospital of Soochow University, 58 South Changsheng Street, Taicang 215400, Jiangsu, PR China. Tel: +86-512-53610208; Fax: +86-512-53610208; E-mail: ggj1326@163.com

References

[1] Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66: 115-132.

[2] Kadara H, Kabbout M and Wistuba II. Pulmonary adenocarcinoma: a renewed entity in 2011. Respirology 2012; 17: 50-65.

[3] Herbst RS, Heymach JV and Lippman SM. Lung cancer. N Engl J Med 2008; 359: 1367-1380.

[4] Gold KA, Kim ES, Lee JJ, Wistuba II, Farhangfar CJ and Hong WK. The BATTLE to personalize lung cancer prevention through reverse migration. Cancer Prev Res (Phila) 2011; 4: 962-972.

[5] Siegel R, DeSantis C, Virgo K, Stein K, Mariotto A, Smith T, Cooper D, Gansler T, Lerro C, Fedewa S, Lin C, Leach C, Cannady RS, Cho H, Scoppa S, Hachey M, Kirch R, Jemal A and Ward E. Cancer treatment and survivorship statistics, 2012. CA Cancer J Clin 2012; 62: 220-241.

[6] Sarkar S, Roy BC, Hatano N, Aoyagi T, Gohji K and Kiyama R. A novel ankyrin repeat-containing gene (Kank) located at 9p24 is a growth suppressor of renal cell carcinoma. J Biol Chem 2002; 277: 36585-36591.

[7] Weng Z, Shang Y, Yao D, Zhu J and Zhang R. Structural analyses of key features in the KANK1.KIF21A complex yield mechanistic insights into the cross-talk between microtubules and the cell cortex. J Biol Chem 2018; 293: 215-225.

[8] Kakinuma N, Zhu Y, Wang Y, Roy BC and Kiyama R. Kank proteins: structure, functions and diseases. Cell Mol Life Sci 2009; 66: 2651-2659.

[9] Kariri YA, Joseph C, Kurozumi S, Toss MS, Alsaleem M, Raafat S, Mongan NP, Aleskandarany MA, Green AR and Rakha EA. Prognostic significance of KN motif and ankyrin repeat domains 1 (KANK1) in invasive breast cancer. Breast Cancer Res Treat 2020; 179: 349-357.

[10] Huang WG, Cheng AL, Chen ZC, Peng F, Zhang PF, Li MY, Li F, Li JL, Li C, Yi H, Li XH, Yi B and Xiao ZQ. Targeted proteomic analysis of 14-3-3sigma in nasopharyngeal carcinoma. Int J Biochem Cell Biol 2010; 42: 137-147

[11] Fan H, Tian H, Cheng X, Chen Y, Liang S, Zhang Z, Liao Y and Xu P. Aberrant Kank1 expression regulates YAP to promote apoptosis and inhibit proliferation in OSCC. J Cell Physiol 2020; 235: 1850-1865.

[12] Chen T, Wang K and Tong X. In vivo and in vitro inhibition of human gastric cancer progress by upregulating Kank1 gene. Oncol Rep 2017; 38: 1663-1669.

[13] Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U and Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984; 133: 1710-1715.

[14] Dowsett M, Nielsen TO, A'Hern R, Bartlett J, Coombes RC, Cuzick J, Ellis M, Henry NL, Hugh JC, Lively T, McShane L, Paik S, Penault-Llorca F, Prudkin L, Regan M, Salter J, Sotiriou C, Smith IE, Viale G, Zujewski JA, Hayes DF; International Ki-67 in Breast Cancer Working Group. Assessment of ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group. J Natl Cancer Inst 2011; 103: 1656-1664.

[15] Luo Y, Ren F, Liu Y, Shi Z, Tan Z, Xiong H, Dang Y and Chen G. Clinicopathological and prognostic significance of high Ki-67 labeling index in hepatocellular carcinoma patients: a meta-analysis. Int J Clin Exp Med 2015; 8: 10235-10247.

[16] Pezzilli R, Partelli S, Cannizzaro R, Pagano N, Crippa S, Pagnanelli M and Falconi M. Ki-67 prognostic and therapeutic decision driven marker for pancreatic neuroendocrine neoplasms (PNENs): a systematic review. Adv Med Sci 2016; 61: 147-153.

[17] Richards-Taylor S, Ewings SM, Jaynes E, Tilley C, Ellis SG, Armstrong T, Pearce N and Cave J. The assessment of Ki-67 as a prognostic marker in neuroendocrine tumours: a systematic review and meta-analysis. J Clin Pathol 2016; 69: 612-618.

[18] In: Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL and Trotti A, editors. AJCC cancer staging manual. 7th edition. New York, NY: Springer; 2010.

[19] Xu ST, Ma YC, Wang CH, Xu Y and Gu GJ. Prognostic and clinicopathologic significance of AEG-1/MTDH and E-cadherin expression in human gallbladder carcinoma. Int J Clin Exp Pathol 2018; 11: 6025-6031.

[20] Kakinuma N, Roy BC, Zhu Y, Wang Y and Kiyama R. Kank regulates RhoA-dependent formation of actin stress fibers and cell migration via 14-3-3 in PI3K-Akt signaling. J Cell Biol 2008; 181: 537-549.

[21] Roy BC, Kakinuma N and Kiyama R. Kank attenuates actin remodeling by preventing interaction between IRSp53 and Rac1. J Cell Biol 2009; 184: 253-267.

[22] Guo X, Fan W, Bian X and Ma D. Upregulation of the Kank1 gene-induced brain glioma apoptosis and blockade of the cell cycle in G0/G1 phase. Int J Oncol 2014; 44: 797-804.

[23] Cui Z, Shen Y, Chen KH, Mittal SK, Yang JY and Zhang G. KANK1 inhibits cell growth by inducing apoptosis through regulating CXXC5 in human malignant peripheral nerve sheath tumors. Sci Rep 2017; 7: 40325.