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Original Article

GRK6 over-expression predicts poor outcomes in patients with lung adenocarcinoma

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Abstract: Objective is to investigate the expression and potential roles of G protein-coupled receptor kinase 6 (GRK6) in lung adenocarcinoma patients. Immunohistochemistry was performed to determine GRK6 expression in 196 lung adenocarcinoma samples. And the relationship between expression and clinicopathological features was analyzed. Lung adenocarcinoma patients with higher GRK6 expression had poor outcomes. And GRK6 high expression was associated with metastasis and relapse. GRK6 overexpression may be a significant independent prognostic factor of lung adenocarcinoma.

Keywords: GRK6, lung adenocarcinoma, prognosis

Introduction

Lung adenocarcinoma is one of commonest malignant tumors, whose pathogenesis is still not complete clear. And its morbidity is rising year by year in the whole world. GRK6 is an important protein kinase in human, and it participate many pathological process of different kinds of disease [1, 2]. GRK6 belongs to the seven-member G-protein coupled receptor kinase family [3] and it regulates diverse cellular functions ranging from metabolism to growth. GRK6 can regulate the activity of the chemokine receptor by phosphorylation [4, 5]. Previous research has reported that the lack of GRK6 can prevent cancer progression [6]. However, the expression in lung adenocarcinoma has not been reported. This study is to explore the expression and the function of GRK6 in lung adenocarcinoma and to analyze the correlation between GRK6 expression and the clinicopathological features.

Materials and methods

Patients and clinical data

A total of 195 lung adenocarcinoma samples were collected from patients admitted from 2006 to 2008, and all samples were confirmed by pathological histological analysis (**Table 1**). It included 89 T1, 90 T2, 10 T3 and 5 T4. 90 patients have lymph nodes metastasis. And 11 patients had distant organs metastasis. 128 patients were in I~II pathologic stage and the others were in III to IV pathologic stage. 65 patients had relapse or metastasis. Besides, 109 patients have chemoradiotherapy history. Among these 195 samples 173 patients have follow-up information. 100 of them were still alive until January 2014. Other 73 patients were dead.

GRK6 antibody from rabbits and β-actin antibody from rats were from Santa Cruz Company, chemiluminescent solution was supplied by CST company, SP kit was from Dako, and the other chemical reagent was analytical reagent made in china.

Immunohistochemistry

Paraffin-embedded tissue sections (3 mm) were subjected to immunohistochemistry. Tissue sections were made and used to assess the difference in histology and GRK6 expression. Sections were deparaffinised by xylene, rehydrated in graded concentrations of ethanol, and were pretreated in an autoclave at 121°C

Table 1. Relationship between clinical pathologic features and GRK expression in lung adenocarcinoma tissue

Clinical pathologic feature	N	High expression	Low expression	Pearson Chi-square	P
T classification				6.001	0.112
T1	89	42	47		
T2	90	48	42		
T3	10	8	2		
T4	5	4	1		
N classification				0.002	0.965
N1	90	46	44		
N0	105	54	51		
M classification				4.351	0.037
M1	11	9	2		
M0	184	91	93		
Pathologic stages				0.16	0.689
I~II	128	64	64		
III~IV	66	35	31		
Differentiation degree				2.525	0.471
Moderate-well~well	18	7	11		
Poorly-moderate~moderate	143	76	67		
Poorly	26	12	14		
Undifferentiated	1	1	0		
Relapse & metastasis				5.429	0.02
Yes	65	41	24		
No	130	59	71		
Treatment history				3.578	0.059
Have chemoradiotherapy	109	61	48		
No chemoradiotherapy	48	19	29		
Alive or Death				7.193	0.007
Alive	100	41	59		
Death	73	45	28		

for 15 min in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubation for 30 min with 0.3% hydrogen peroxide in methanol. Non-specific binding sites were blocked in phosphate-buffered saline (PBS, pH 7.4) with 10% normal rabbit serum. The sections were then incubated with primary rabbit monoclonal antibody against GRK6 (1:50) for a night at 4°C. Rewarming in the 37°C caloratat for 30 minutes. After washing with PBS, sections were loaded with secondary antibody (50l) for 30 min in the 37°C caloratat. Then the sections were incubated with Streptomycin avidin-peroxide polymerase for 30 min in the 37°C caloratat. After washing with PBS, drop 100l DAB to get color. The sections were counterstained with haematoxylin for 1-2 minutes, dehydrated in graded concentrations of ethanol, transparented and fixed by neutral xylene balata. GRK6 expression was observed and analyzed under light microscope, and was presented by positive cell percentage.

Statistical analysis

All statistical analyses were carried out with SPSS 13.0 statistical software. Chi-square test was used for GRK6 expression. The survival rate acquired from follow-up visit was analyzed with Kaplan-Meier survival curve and Single variable Log rank test. All the tests were two sides and statistical significance was set at $P< 0.05$. In addition, a multivariate analysis was conducted using the Cox proportional hazard model.

Results

GRK6 expression in lung adenocarcinoma and relationship between GRK6 and patients clinical characteristics

Relationship between clinical pathologic features and GRK expression in lung adenocarcinoma tissue was shown in **Table 1**. The expression of GRK6 in lung adenocarcinoma was shown in **Figure 1**. According to the Chi-square test, immunohistochemical result shows that the high expression of GRK6 is associated with poor prognosis (P=0.007). It has statistic significance between GRK6 expression and distant organs metastasis (P=0.037). Besides, the expression of GRK6 is associated with relapse and distant metastasis (P=0.02).

Univariate analysis of clinical and pathologic characteristics

The univariate analysis of clinic and pathologic characteristics was summarized in **Table 2**.

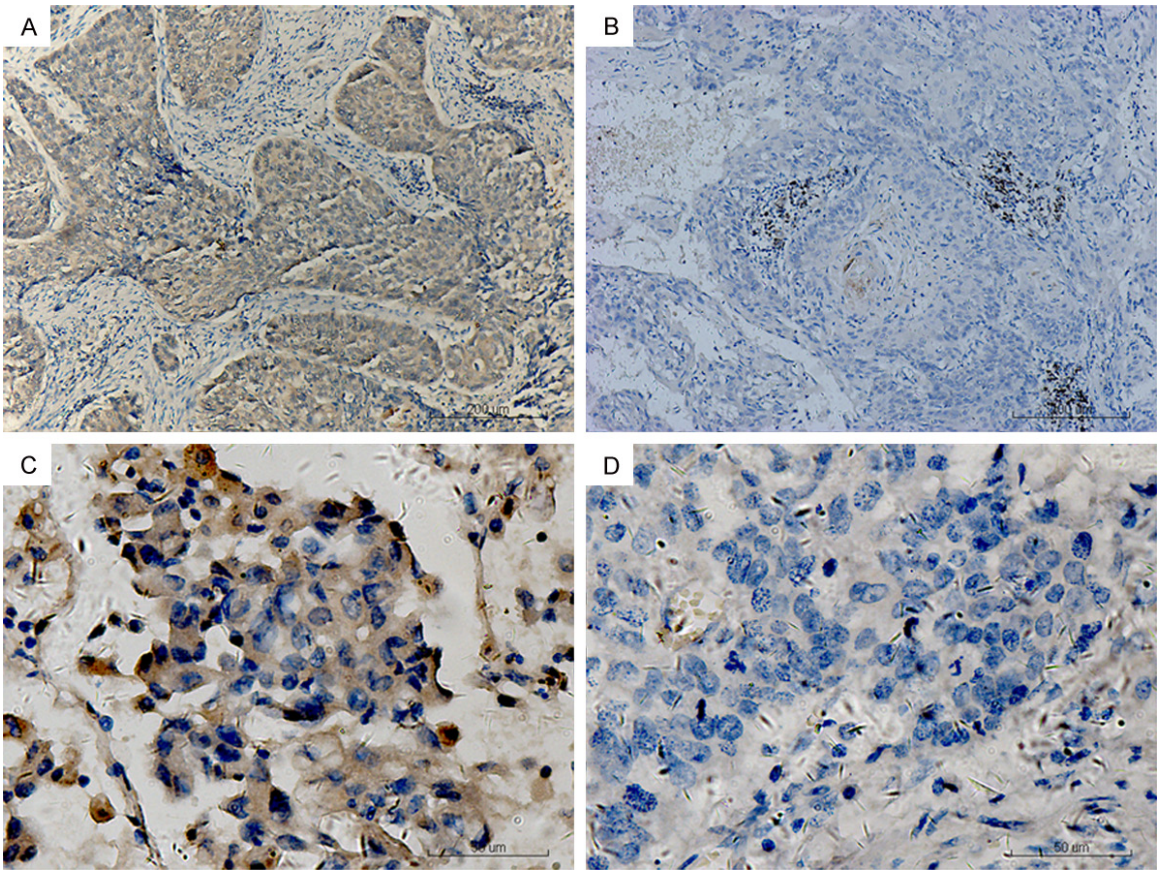

Figure 1. A. The expression of GRK6 is positive at ×100 magnification (10× objective lens). B. The expression of GRK6 is negative at ×100 magnification (10× objective lens). C. The expression of GRK6 is positive at ×400 magnification (40× objective lens). D. The expression of GRK6 is negative at ×400 magnification (40× objective lens).

Survival data of 173 cases was collected from 195 cases, including 86 patients with high GRK6 expression and 87 patients with low GRK6 expression. 5-year overall survival rate of high GRK6 expression was 50%. And the low GRK6 expression group was 68%. According to the statistic analysis, the GRK6 expression (P=0.011), chemoradiotherapy treatment (P= 0.002), TNM classification (P<0.001), pathologic stages (P<0.001) and differentiation degree (P<0.001) were associated with outcome. And the Kaplan-Meier survival curve of GRK6 expression was shown in **Figure 2** (P=0.011).

Multivariate analysis of clinical and pathologic characteristics

The multivariate analysis of clinic and pathologic characteristics was summarized in **Table 3**. Using the Cox proportional hazard model, we analysis the features that showed statistic significance in univariate analysis. The pathologic stages (Hazard Ratio, HR=0.438, P=0.002) and GRK6 expression (HR=0.526, P=0.016) showed statistic significance. This result indicated that the expression of GRK6 might be an independent prognostic factor of lung adenocarcinoma. On the other hand, the chemoradiotherapy treatment history did not have statistic significance (HR=0.673, P=0.287).

Discussion

Lung adenocarcinoma has high malignant degree and morbidity, whose postoperative recurrence and metastasis are the significant factors of prognosis. Many cases fail to be diagnosed until the late stage and losing the chance to be treated. It is urgent to find a significant target to help diagnose and therapy in the early stage. The exploration of the metastasis is the pre-requisite way to go. While the metastasis of the lung adenocarcinoma is a multiple and complex stage process, the cancer cells abate

Table 2. Survival Analysis--Univariate Analysis of Clinical and Pathologic Characteristics

Features	n	5-Year OS (%)	P
T classification			<0.001
T1	81	67	
T2	77	57	
T3	9	11	
T4	5	60	
N classification			<0.001
N1	83	41	
N0	90	76	
M classification			<0.001
M1	10	10	
M0	163	62	
Pathologic stages			<0.001
I~II	113	71	
III~IV	59	37	
Differentiation degree			<0.001
Moderate-well~well	16		
Poorly-moderate~moderate	128	54	
Poorly	23	52	
Undifferentiated	1		
Relapse & metastasis			<0.001
Yes	63	22	
No	110	80	
Treatment history			0.002
Have chemoradiotherapy	107	51	
No chemoradiotherapy	48	79	
GRK6 expression			0.011
High expression	86	50	
Low expression	87	68	

adhesivity and enhance athletic ability in this process. To achieve this ability, substances such as matrix metalloproteinase are secreted to degrade extracellular matrix and as a result the tumor cells can cross through the basement membrane and extracellular matrix [7].

GRK6 is a member of the seven-member G-protein coupled receptor kinase family (GRKs) [3]. GRKs mediate phosphorylation-dependent desensitization of the G-protein coupled receptors (GPCRs). However, emerging evidence shows that the substrates of GRKs are far beyond the GPCRs [8]. By marketing GPCR phosphorylation, GRK can improve the affinity between GPCR and arrestins, the formed heterotrimer then induce GPCR desensitization and endocytosis, as a result the downstream signal transduction is blocked [9]. However, emerging evidence shows that the substrates of GRKs are far beyond the GPCRs. GRK can also combine with other kinds of receptors, such as Transforming growth factor receptor (TGFR), Epidermal growth factor receptor (EGFR), Platelet-derived growth factor receptor (PDGFR), Insulin-like growth factor receptor (IGFR) and so on [10]. The member of GRKs contains a high conserved regulator of G protein signaling protein domain, a Ser-Thr kinase domain which is similar to AGC protein kinase domain, and a carboxyl terminal which is affected with cell membrane [11]. The GRK6 is located in the membrane before the activators activate GPCRs. Through the palmitoylation of cysteine terminal, the GPCR are undergoing phosphorylation and endocytosis [12].

Previous research shows that the GRKs have closely relationship with the movement and migration of the cells. For example, GRK2 participates in cell movement and cell migration of the integrin [13]. GRK2 can phosphorylate MAPK and to block the downstream signal transduction. Besides, the TGFR can increase the expression of the GRK2, and then GRK2 can phosphorylate Smad2 and Smad3, and eventually as a result to prevent the transcription of the target gene induced by TGFR [14]. GRK6 interacted with GRK-interacting protein (GIT1), and adjust the cell adhere and cytoskeleton reconstruction [15]. However, the study of the relationship between GRK6 and cell migration and movement is limited. It still needs more exploration.

In conclusion, this study finds that GRK6 high expression in lung adenocarcinoma, and might related to the migration of the tumor cells. The expression of GRK6 may help diagnose lung adenocarcinoma in the early stage and to evaluate the prognosis of the patients. The molecular biochemistry mechanism still needs deeper exploration.

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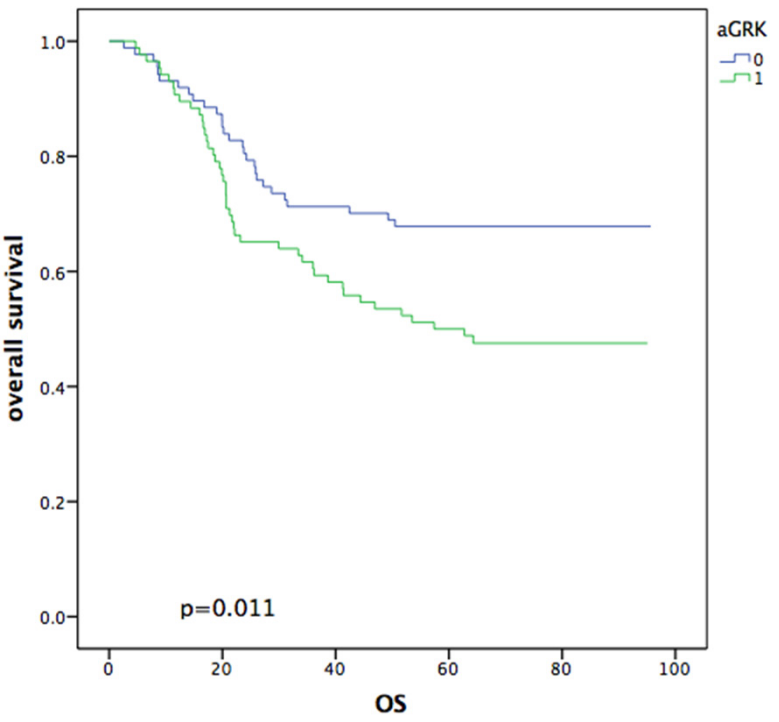

Figure 2. Patients with high GRK6 expression had shorter 5-year OS than low GRK6 expression.

Table 3. Multivariate Prognostic Analysis

Factors	Category	Hazard ratio	95% confidence interval	P
GRK6	+	0.526	0.312-0.887	0.016
	-			
Chemoradiotherapy	+	0.673	0.325-1.395	0.287
	-			
Differentiation degree		0.812	0.425-1.549	0.527
	poor-moderate~well			
	poor~undifferentiated			
Pathologic stages	I~II	0.438	0.257-0.438	0.002
	III~IV			

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Disclosure of conflict of interest

None.

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References

[1] Ahmed MR, Bychkov E, Kook S, Zurkovsky L, Dalby KN, Gurevich EV. Overexpression of GRK6 rescues L-DOPA-induced signaling abnormalities in the dopamine-depleted striatum of hemiparkinsonian rats. Exp Neurol 2015; 266: 42-54.

[2] Tiedemann RE, Zhu YX, Schmidt J, Yin H, Shi CX, Que Q, Basu G, Azorsa D, Perkins LM, Braggio E, Fonseca R, Bergsagel PL, Mousses S, Stewart AK. Kinome-wide RNAi studies in human multiple myeloma identify vulnerable kinase targets, including a lymphoid-restricted kinase, GRK6. Blood 2010; 115: 1594-604.

[3] Willets JM, Challiss RA and Nahorski SR. Non-visual GRKs: are we seeing the whole picture? Trends Pharmacol Sci 2003; 24: 626-33.

[4] Vroon A, Heijnen CJ, Raatgever R, Touw IP, Ploemacher RE, Premont RT, Kavelaars A. GRK6 deficiency is associated with enhanced CXCR4-mediated neutrophil chemotaxis in vitro and impaired responsiveness to G-CSF in vivo. J Leukoc Biol 2004; 75: 698-704.

[5] Busillo JM, Armando S, Sengupta R, Meucci O, Bouvier M, Benovic JL. Site-specific phosphorylation of CXCR4 is dynamically regulated by multiple kinases and results in differential modulation of CXCR4 signaling. J Biol Chem 2010; 285: 7805-17.

[6] Chen Y, Lu B, Yang Q, Fearns C, Yates JR 3rd, Lee JD. Combined integrin phosphoproteomic analyses and small interfering RNA--based functional screening identify key regulators for

cancer cell adhesion and migration. Cancer Res 2009; 69: 3713-20.
[7] Martin TA, Mason MD and Jiang WG. Tight junctions in cancer metastasis. Front Biosci (Landmark Ed) 2011; 16: 898-936.
[8] Ribas C, Penela P, Murga C, Salcedo A, García-Hoz C, Jurado-Pueyo M, Aymerich I, Mayor F Jr. The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signaling. Biochim Biophys Acta 2007; 1768: 913-22.
[9] Lymperopoulos A and Bathgate A. Pharmacogenomics of the heptahelical receptor regulators G-protein-coupled receptor kinases and arrestins: the known and the unknown. Pharmacogenomics 2012; 13: 323-41.
[10] Hupfeld CJ and Olefsky JM. Regulation of receptor tyrosine kinase signaling by GRKs and beta-arrestins. Annu Rev Physiol 2007; 69: 561-77.
[11] Mushegian A, Gurevich VV and Gurevich EV. The origin and evolution of G protein-coupled receptor kinases. PLoS One 2012; 7: e33806.
[12] Stoffel RH, Inglese J, Macrae AD, Lefkowitz RJ, Premont RT. Palmitoylation increases the kinase activity of the G protein-coupled receptor kinase, GRK6. Biochemistry 1998; 37: 16053-9.
[13] Penela P, Ribas C, Aymerich I, Mayor F Jr. New roles of G protein-coupled receptor kinase 2 (GRK2) in cell migration. Cell Adh Migr 2009; 3: 19-23.
[14] Ho J, Cocolakis E, Dumas VM, Posner BI, Laporte SA, Lebrun JJ. The G protein-coupled receptor kinase-2 is a TGFbeta-inducible antagonist of TGFbeta signal transduction. Embo J 2005; 24: 3247-58.
[15] Premont RT, Claing A, Vitale N, Freeman JL, Pitcher JA, Patton WA, Moss J, Vaughan M, Lefkowitz RJ. beta2-Adrenergic receptor regulation by GIT1, a G protein-coupled receptor kinase-associated ADP ribosylation factor GTPase-activating protein. Proc Natl Acad Sci U S A 1998; 95: 14082-7.