JBUON 2017; 22(6): 1441-1446
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Downregulation of guanine nucleotide-binding protein beta 1 (GNB1) is associated with worsened prognosis of clear-cell renal cell carcinoma and is related to VEGF signaling pathway

Chen Chen, Chi Huang, Min Liu, Junhua Zheng

Department of Urology, Shanghai Tenth People's Hospital, Tongji University, Shanghai, 200072, China

Summary

Purpose: *Clear-cell renal cell carcinoma (ccRCC) is characterized by genetic abnormalities, while the role of Guanine Nucleotide-Binding Protein Beta 1 (GNB1) in ccRCC has not been studied. We thus aimed to evaluate the expression and prognostic value of GNB1 in ccRCC.*

Methods: *A two-stage study (exploration and validation) was conducted using in silico and immunohistochemical (IHC) scoring of ccRCC samples from our institute, to evaluate the association between GNB1 expression and clinicopathological parameters of ccRCC patients. Pathway analyses were performed for genes coexpressed with GNB1 using the KOBAS platform to profile the function of GNB1 and IHC validation.*

Results: *In the exploration stage, data from TCGA ccRCC dataset were reproduced, which contained 537 patients with ccRCC and found that downregulation of GNB1 was significantly associated with worse prognosis. IHC staining from the Human Protein Atlas showed significantly downregulation of GNB1 in ccRCC tissue compared with normal kidney. Pathway analysis showed significantly altered vascular endothelial growth factor (VEGF) signaling pathways among which expressions of 3 genes (WASF2, NRP1, and HIP1) were significantly associated with GNB1 expression, respectively. In the validation stage, included were 80 ccRCC samples and GNB1 expression was scored using IHC positivity. GNB1 expression was negatively associated with tumor stage, lymph node invasion, metastasis, older age, and increased tumor grade. Female gender and receiving neoadjuvant therapy were also associated with decreased GNB1 expression. The expressions of WASF2, NRP1 and HIP1 were also studied and found that they were significantly associated with GNB1.*

Conclusion: *GNB1 was downregulated in ccRCC. Decreased GNB1 expression was associated with worsened disease characteristics and prognosis. GNB1 was related with VEGF signaling in ccRCC, implying a therapeutic potential of this factor.*

Key words: *GNB1, prognosis, renal cell carcinoma, VEGF*

Introduction

Clear-cell renal cell carcinoma (ccRCC) is the predominant pathological subtype of renal cell carcinoma, which is characterized by unique genetic and genomic alterations [1]. Loss of heterozygosity (LOH) and mutation of genes on 3p are the truncal genetic events in ccRCC [2]. There are 4 significantly mutated genes in ccRCC, namely VHL, PBRM1, SETD2, and BAP1 [3]. Though the latter 3 genes participate in chromatin remodeling, the striking and almost unanimous characteristic of ccRCC is abnormally activated hypoxic signaling originating from impaired von Hippel-Lindau (VHL) function that incurs accumulated hypoxia-inducible factors (HIFs). The HIFs further induce release of VEGF, which promotes hypervascularization [4]. Anti-angiogenic, namely anti-VEGF therapy,

Correspondence to: Junhua Zheng, MD. Department of Urology, Shanghai Tenth People's Hospital, Tongji University, Shanghai, 200072, China.
Tel and Fax: +86 21 66305158, E-mail: drurology@126.com
Received: 21/03/2017; Accepted: 17/04/2017

currently stands as first-line systemic therapy of metastatic ccRCC [5]. Nonetheless, all anti-angiogenic therapies would eventually incur resistance and VEGF-resistant patients would soon succumb due to uncurbed disease [6]. Therefore, identification of novel tumor markers within angiogenic or VEGF signaling pathway would provide additive therapeutic interests to the current anti-VEGF agents.

GNB1 belongs to heterotrimeric guanine nucleotide-binding proteins (G proteins), which integrate signals between receptors and effector proteins, and are composed of an alpha, beta, and gamma subunit. These subunits are encoded by families of related genes. GNB1 encodes a beta subunit. Beta subunits are important regulators of alpha subunits, as well as of certain signal transduction receptors and effectors [7]. There has been increasing evidence showing critical role of signal transduction in cancer development [8]. However, the role of GNB1 in cancer has not been elucidated and its role in RCC is not yet reported. Genetic alteration of GNB1 was shown to play a role in some cancers. In breast cancer, upregulation of GNB1 expression is associated with advanced tumor stage and worsened prognosis and is related to mTOR signaling [9]. In myeloid and B cell neoplasms, recurrent mutation of GNB1 is associated with resistance to PI3K-mTOR inhibitor BEZ235 [10]. These results indicate that the role of GNB1 in cancer may be alteration type- and tissue context- dependent.

Therefore, in the current study we investigated the expression of GNB1 in ccRCC using a 2-stage strategy. The exploration stage is an *in silico* analysis of high throughput genetic and protein databases. The validation stage consists of our own collection of RCC tissues which were evaluated for GNB1 expression using immunohistochemistry (IHC) to validate the findings in the exploratory stage.

Methods

Exploration stage

Reproduction of the Cancer Genome Atlas (TCGA) dataset

The clear cell kidney cancer subset (KIRC) of TCGA was reproduced to study the expression of GNB1 in ccRCC and its prognostic merit using the cBioPortal platform [1,11,12]. Using the cBioPortal platform, we downloaded RNA seq data of GNB1 and clinicopathological data of all 538 patients with ccRCC. The expression level of GNB1 was determined using the OncoPrint function of cBioPortal online. List of genes coexpressed with GNB1 detected using RNA seq was generated using the Coexpression function of cBioPortal online, which automatically calculated the correlation coefficient (R) using the Pearson test. We subjected genes passing the ±40 of coefficient R to the KOBAS 3.0 platform for functional annotation [13,14]. Only KEGG Pathway and Reactome datasets were allowed for annotation.

Reproduction of the Human Protein Atlas dataset

The expression of GNB1 in normal and cancerous kidney tissue was evaluated semi-quantitatively using the Human Protein Atlas platform [15-18]. The IHC staining intensity was graded as follows: 0 for 0–5% of tumor cells stained, 1 for 6–20% of cells stained, 2 for 21–50% of cells stained and 3 for > 50% of cells stained. The staining intensity was graded as follows: 1 for light yellow, 2 for dark yellow and 3 for brown. Sum of IHC intensity and intensity represented the final quantification of each sample: 0 for negative (1-2), 1 for mild (3), 2 for moderate (4), and 3 for strong (5-6).

Statistics

The SPSS 22.0 and Prism Graphpad 6.0 software were used for statistical analyses. The differences of clinicopathological parameters between downregulated and unchanged GNB1 expression were compared using the Chi-square and Fisher's exact tests. Age difference between groups with different GNB1 expression was compared with Student's t-test. A p value <0.05 was accepted as statistically significant.

Validation stage

Patients and samples

In the validation stage, 80 ccRCC samples from patients undergoing partial nephrectomy, radical nephrectomy, or cytoreductive nephrectomy at our institute were included. The clinicopathological parameters were collected and reviewed retrospectively. The TNM system was used for staging and the Fuhrman four-tier system was used for nuclear grading. The study was approved by local institutional review board.

Immunohistochemistry

A standard hematoxylin and eosin staining procedure was performed in all samples [19-22]. Formalin-fixed, paraffin-embedded tissue samples were sliced consecutively at 4 μm and were mounted on polylysine-coated glass slides. Endogenous peroxidase of deparaffinized sections was blocked through incubation with 3% hydrogen peroxide for 15 min. The samples were then deparaffinized, with gradient rehydration in ethanol. The following antibodies were used for IHC staining: GNB1 (Abcam), huntingtin interacting protein 1 (HIP1) (Santa Cruz Biotechnology), Neuropilin 1 (NRP1) (Abcam), and WAS protein family member 2 (WASF2) (Abcam). Specific dilution of each enzyme was per manufacturer's protocol. We used DAB (diaminobenzidine tetrahydrochloride) solution for color developing and all slides were finalized by counterstaining with Mayer's hematoxylin blue. Positive and negative controls for all enzyme labelers were referenced using the Human Protein Atlas platform. The scoring system for GNB1 was aforementioned. Semi-quantification for

HIP1, NRP1, and WASF2 was as follows: 0 for 0-10% of tumor cells stained, 1 for 11-25% of cells stained, 2 for 26–50% of cells stained and 3 for >50% of cells stained. The intensity scoring and final IHC scoring method was the same as for GNB1.

Statistics

The SPSS 22.0 and Prism Graphpad 6.0 software were used for statistical analyses. The Mann-Whitney U test was used to compare expressional differences between 2 groups. Expressional differences among > 2 groups were analyzed by one-way ANOVA test. Correlation was analyzed by the Pearson's x^2 test. A p value <0.05 was accepted as statistically significant.

Results

In the exploratory stage, we found that downregulation of GNB1 occurred in 10.2% of ccRCC cases. With dichotomized determination (downregulation vs unchanged), the distribution of clinicopathological parameters were summarized in Table 1. Downregulation of GNB1 was not associated with TNM stage, age, gender, Fuhrman grade, or whether neoadjuvant therapy was applied or not (Table 1). Protein level of GNB1 was significantly lower in RCC tissue compared with normal kidney tissue (Figure 1A). Cropped representative images can be accessed via http://www.proteinatlas.org/ENSG00000078369-GNB1/tissue/kidney#imid_14117958 for normal kidney, and via http://www.proteinatlas.org/ENSG00000078369-GNB1/cancer/tissue/renal+cancer#img for RCC tissue. Expressions of a total of 227 genes were correlated with GNB1 expression in ccRCC samples with a Pearson's R of > 0.4. Pathway analysis of those genes revealed that signaling by VEGF from Reactome dataset was among the top 10 significantly altered pathways. Three genes within the VEGF

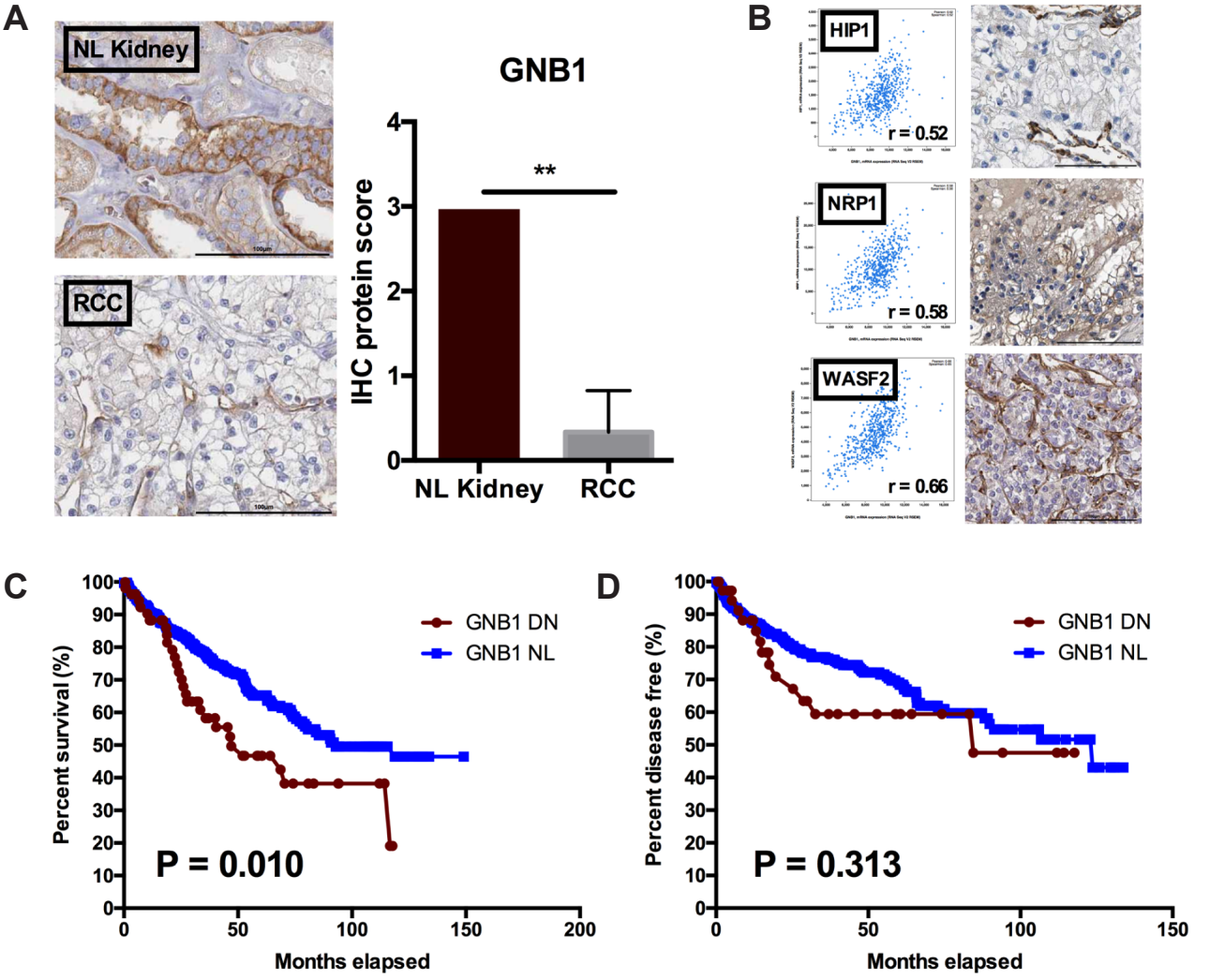

Figure 1. Exploration stage of the study reproducing TCGA and Human Protein Atlas datasets showing: **(A)** significant downregulation of GNB1 expression in renal cell carcinoma (RCC) compared with normal (NL) kidney tissue; and **(B)** genes within VEGF signaling pathway were coexpressed with GNB1 in RCC; Kaplan-Meier plotting showing cases with GNB1 downregulation (DN) had significantly worsened prognosis **(C)** and unaltered disease free survival compared with cases with unchanged GNB1 expression (NL) **(D)** (**p < 0.01).

signaling pathway with strongest correlation with GNB1 expression were HIP1 (Pearson score=0.52), NRP1 (Pearson score=0.58), and WASF2 (Pearson score=0.66) (Figure 1B). Cropped representative images can be accessed via http://www.proteinatlas.org/ENSG00000127946-HIP1/cancer/tissue/renal+cancer#img for HIP1, via http://www.proteinatlas.org/ENSG00000099250-NRP1/cancer/tissue/renal+cancer#img for NRP1, and via http://www.proteinatlas.org/ENSG00000158195-WASF2/cancer/tissue/renal+cancer#img for WASF2. Cases with GNB1 downregulation showed significantly worsened prognosis (Figure 1C). However, GNB1 downregulation was not associated with progression-free survival (Figure 1D).

The clinicopathological parameters were summarized in Table 1. We found that lowered GNB1 expression level in terms of IHC score was significantly associated with increased T stage, nodal involvement, and metastasis (Table 1). GNB1 expression also showed a significant correlation with age (Table 1). Cancers from female patients showed significantly higher GNB1 expression than the male counterparts (Table 1). Decreased GNB1 expression was also associated with higher Fuhrman grade (Table 1). Patients who underwent neoadjuvant therapy had significantly decreased GNB1 level (Table 1). In the correlation analysis, we found that GNB1 expression was significantly correlated with expressions of WASF2, NRP1, and HIP1, respectively (Table 2).

Discussion

Identification of novel prognostic markers for RCC is of great importance. Herein, we report that GNB1 is downregulated in RCC and downregulation of GNB1 is associated with worsened prognosis. The more aggressive phenotype associated with GNB1 downregulation is also validated externally using samples from our own institute. The difference in the association between GNB1 expression and clinicopathological parameters in the exploration and validation stages could be due

Table 1. Expression of Guanine Nucleotide-Binding Protein Beta 1 (GNB1) in association with clinicopathological parameters of patients with clear-cell renal cell carcinoma

Parameter	*Exploration*				*Validation*			
	Breakdown	*GNB1 Expression (n)*		*p*	*Breakdown*	*n*	*GNB1 Expression (M ± SD)*	*p*
		Down	*NL*					
T	T1	23	252	0.126	T1	37	2.14 ± 0.419	< 0.001
	T2	6	63		T2	24	1.21 ± 0.588	
	T3	26	156		T3	14	0.357 ± 0.497	
	T4	0	11		T4	5	0.400 ± 0.894	
N	N0	30	209	0.703	N0	65	1.72 ± 0.673	< 0.001
	N1	1	16		N1	15	0.200 ± 0.561	
M	M0	42	383	0.426	M0	75	1.51 ± 0.844	0.013
	M1	10	69		M1	5	0.400 ± 0.894	
Age (years)		62.09 ± 12.21	60.39 ± 12.14	0.327			r = 0.446	< 0.001
Gender	Male	34	312	0.659	Male	44	1.70 ± 0.765	0.006
	Female	21	170		Female	36	1.11 ± 0.919	
Grade	I	1	13	0.164	I	18	2.28 ± 0.461	< 0.001
	II	17	212		II	44	1.55 ± 0.627	
	III	28	180		III	14	0.429 ± 0.646	
	IV	6	72		IV	4	0.0 ± 0.0	
Neoadjuvant Tx	No	55	464	0.24	No	73	1.55 ± 0.817	< 0.001
	Yes	0	18		Yes	7	0.286 ± 0.756	

T= tumor stage, N= lymph node, M= metastasis, Tx= treatment, n= number, NL= normal, M ± SD= mean ± standard deviation

Table 2. Correlation between expressions of in guanine nucleotide-binding protein beta 1 (GNB1), WAS protein family member 2 (WASF2), neuropilin 1 (NRP1), huntingtin interacting protein 1 (HIP1) in clear-cell renal cell carcinoma

	GNB1 vs. WASF2	*GNB1 vs. NRP1*	*GNB1 vs. HIP1*
Pearson r	0.91	0.894	0.868
95% confidence interval	0.862 to 0.941	0.839 to 0.931	0.801 to 0.914
R square	0.827	0.799	0.753
P (two-tailed)	< 0.001	< 0.001	< 0.001

to the following reasons: First, different stratification systems were adopted in the 2 stages. In the exploration stage, a dichotomized system was used and in the validation stage the expression was scored as a continuous variant; Second, our retrospective validation was inevitably subjected to selection bias. For instance, in our cohort, female patients tended to have disease at earlier stage and almost all T4 node-positive and metastatic cases were men. Also, patients subjected to neoadjuvant therapy varied vastly by regime, course and duration, which made the significant difference of GNB1 expression possibly false-positive. Nonetheless, it remains intriguing that none of the major prognostic contributors (e.g. TNM, and Fuhrman grade) was significantly associated with GNB1 downregulation, which however still impacted substantially overall survival. In the Kaplan-Meier plots, one can see that at about 30 months of follow-up, the declines of overall survival and progression-free survival in the GNB1 dowregulated cohort were corresponding, while thereafter the progression-free survival remained comparable to the counterpart with overall survival continuously declining. This pattern supports a delayed prognostic effect, which is typical for an immunomodulatory factor. Via literature search on the very limited reports on the role of GNB1 in all types of cancers, we suggest that the oncogenic effect in GNB1 is cancer- and gene alteration-dependent. In the current study, we have revealed for the first time that downregulation of GNB1 in ccRCC is associated with worsened prognosis and this effect could be exerted via the following pathways:

First, upon knowledge-based speculation, GNB1 could be affecting immunomodulation of ccRCC. Chemokines are required for leukocyte recruitment and appropriate host defense, and act through G protein-coupled receptors, which induce downstream signaling leading to integrin activation. Recent evidence showed that all isoforms of GNBs (GNB1, GNB2, GNB4, and GNB5) are required for activation of lymphocyte function-associated antigen 1 (LFA-1) [23]. Specifically, downregulation of GNB1 leads to a significant impairment of LFA-1 activation. LFA-1 is reported to interact with programmed cell death 1 (PD-1), a negative immumodulator that inactivates tumor infiltrating lymphocytes (TILs) [24]. Anti-PD-1/PD-L1 therapy has now became second-line in the systemic treatments for RCC [25]. The detailed mechanistic analysis of GNB1 in immune response in ccRCC warrants further studies.

Second, we revealed that GNB1 downregulation is associated with VEGF signaling pathway. Significant expressional correlations between GNB1 and key genes within the VEGF pathway were noted. WASF2-regulated actin reorganization might be required for proper cell movement and that a lack of functional WASF2 impairs angiogenesis *in vivo* [26]. In hepatocellular carcinoma, which is also characterized with overexpression of VEGF and hypervasculation, WASF2-Rac1 signaling plays a significant role in angiogenesis [27]. The role of NRP1 in cancer development is vastly reported. NRP1 is believed to exert protumorigenic role both via angiogenic and nonangiogenic pathways [28]. In the angiogenic aspect, VEGF165-induced vascular permeability requires NRP1 for ABL-mediated SRC family kinase activation [29]. HIP1 is an endocytic protein with transforming properties that is involved in a cancer-causing translocation and which is overexpressed in a variety of human cancers [30]. The HIP1-ALK fusion plays a role in non-small-cell lung cancer (NSCLC) [31]. The role of HIP1 in angiogenesis is, however, not clear. In the current study, we have demonstrated that GNB1 is in close association with WASF2, NRP1 and HIP1, not only putting GNB1 in the angiogenic regulation, but also indicating GNB1 as a promising target for drug development. A detailed mechanism how GNB1 mediates angiogenesis in ccRCC warrants profound investigation.

Conclusion

The role of GNB1 in ccRCC has not been reported. In the current study, we found that GNB1 expression was downregulated in ccRCC and lowered GNB1 expression was associated with advanced cancer stage and worsened prognosis. The expression of GNB1 was significantly associated with VEGF signaling in ccRCC. GNB1 is a promising marker for ccRCC and provides therapeutic potential.

Conflict of interests

The authors declare no conflict of interests.

References

1. Cancer Genome Atlas Research N. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature 2013;499:43-9.
2. Hofflin R, Roth W, Sultmann H et al. Intratumoral heterogeneity in renal cell carcinoma. Molecular basis and translational implications. Urologe A 2015;54:800-3.
3. Sato Y, Yoshizato T, Shiraishi Y et al. Integrated molecular analysis of clear-cell renal cell carcinoma. Nat Genet 2013;45:860-7.
4. Ciccarese C, Brunelli M, Montironi R et al. The prospect of precision therapy for renal cell carcinoma. Cancer Treat Rev 2016;49:37-44.
5. Eichelberg C, Vervenne WL, De Santis M et al. SWITCH: A Randomised, Sequential, Open-label Study to Evaluate the Efficacy and Safety of Sorafenib-sunitinib Versus Sunitinib-sorafenib in the Treatment of Metastatic Renal Cell Cancer. Eur Urol 2015;68:837-47.
6. Tannir NM, Schwab G, Grunwald V. Cabozantinib: an Active Novel Multikinase Inhibitor in Renal Cell Carcinoma. Curr Oncol Rep 2017;19:14.
7. Brett M, Lai AH, Ting TW et al. Acute lymphoblastic leukemia in a child with a de novo germline gnb1 mutation. Am J Med Genet A 2017;173:550-2.
8. Qian H, Xuan J, Liu Y, Shi G. Function of G-Protein-Coupled Estrogen Receptor-1 in Reproductive System Tumors. J Immunol Res 2016;2016:7128702.
9. Wazir U, Jiang WG, Sharma AK, Mokbel K. Guanine nucleotide binding protein beta 1: a novel transduction protein with a possible role in human breast cancer. Cancer Genomics Proteomics 2013;10:69-73.
10. Yoda A, Adelmant G, Tamburini J et al. Mutations in G protein beta subunits promote transformation and kinase inhibitor resistance. Nat Med 2015;21:71-5.
11. Gao J, Aksoy BA, Dogrusoz U et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 2013;6:pl1.
12. Cerami E, Gao J, Dogrusoz U et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012;2:401-4.
13. Xie C, Mao X, Huang J et al. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res 2011;39:W316-22.
14. Wu J, Mao X, Cai T, Luo J, Wei L. KOBAS server: a web-based platform for automated annotation and pathway identification. Nucleic Acids Res 2006;34:W720-4.
15. Uhlen M, Fagerberg L, Hallstrom BM et al. Proteomics. Tissue-based map of the human proteome. Science 2015;347:1260419.
16. Uhlen M, Oksvold P, Fagerberg L et al. Towards a knowledge-based Human Protein Atlas. Nat Biotechnol 2010;28:1248-50.
17. Uhlen M, Bjorling E, Agaton C et al. A human protein atlas for normal and cancer tissues based on antibody proteomics. Mol Cell Proteomics 2005;4:1920-32.
18. Ponten F, Jirstrom K, Uhlen M. The Human Protein Atlas--a tool for pathology. J Pathol 2008;216:387-93.
19. Feng C, Ho Y, Sun C, Xia G, Ding Q, Gu B. TFPI-2 expression is decreased in bladder cancer and is related to apoptosis. J BUON 2016;21:1518-23.
20. Wang L, Feng C, Ding G, Zhou Z, Jiang H, Wu Z. Relationship of TP53 and Ki67 expression in bladder cancer under WHO 2004 classification. J BUON 2013;18:420-4.
21. Feng CC, Ding GX, Song NH et al. Paraneoplastic hormones: parathyroid hormone-related protein (PTHrP) and erythropoietin (EPO) are related to vascular endothelial growth factor (VEGF) expression in clear cell renal cell carcinoma. Tumour Biol 2013;34:3471-6.
22. Feng CC, Wang PH, Ding Q et al. Expression of pigment epithelium-derived factor and tumor necrosis factor-alpha is correlated in bladder tumor and is related to tumor angiogenesis. Urol Oncol 2013;31:241-6.
23. Block H, Stadtmann A, Riad D et al. Gnb isoforms control a signaling pathway comprising Rac1, Plcbeta2, and Plcbeta3 leading to LFA-1 activation and neutrophil arrest in vivo. Blood 2016;127:314-24.
24. Arefanian H, Tredget EB, Mok DC et al. Porcine Islet-Specific Tolerance Induced by the Combination of Anti-LFA-1 and Anti-CD154 mAbs Is Dependent on PD-1. Cell Transplant 2016;25:327-42.
25. Motzer RJ, Escudier B, McDermott DF et al. Nivolumab versus Everolimus in Advanced Renal-Cell Carcinoma. N Engl J Med 2015;373:1803-13.
26. Yamazaki D, Suetsugu S, Miki H et al. WAVE2 is required for directed cell migration and cardiovascular development. Nature 2003;424:452-6.
27. Tao Y, Hu K, Tan F et al. SH3-domain binding protein 1 in the tumor microenvironment promotes hepatocellular carcinoma metastasis through WAVE2 pathway. Oncotarget 2016;7:18356-70.
28. Tse BW, Volpert M, Ratther E et al. Neuropilin-1 is upregulated in the adaptive response of prostate tumors to androgen-targeted therapies and is prognostic of metastatic progression and patient mortality. Oncogene 2017;36:3417-27.
29. Fantin A, Lampropoulou A, Senatore V et al. VEGF165-induced vascular permeability requires NRP1 for ABL-mediated SRC family kinase activation. J Exp Med 2017;214:1049-64.
30. Hyun TS, Ross TS. HIP1: trafficking roles and regulation of tumorigenesis. Trends Mol Med 2004;10:194-9.
31. Hong M, Kim RN, Song JY et al. HIP1-ALK, a novel fusion protein identified in lung adenocarcinoma. J Thorac Oncol 2014;9:419-22.