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Systematic Study of von Hippel-Lindau (*VHL*) Gene Pathway in Renal Cell Carcinoma

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Mutation or inactivation of the von Hippel-Lindau (*VHL*) tumor suppressor gene is an early event in the pathogenesis of clear cell renal cell carcinomas (RCCs) and is common in both hereditary and nonhereditary forms.

VHL disease is the most common hereditary renal cancer and is caused by the mutation of germline *VHL* gene. We summarized characteristics of Chinese VHL disease patients: There is a high proportion of novel mutation in Chinese VHL patients. The prevalence of novel mutations without family history was higher in this group of patients, presumably demonstrating the higher prevalence of de novo mutations in *VHL* gene in Chinese VHL disease patients. And genetic anticipation is existed in Chinese VHL patients.

In the research around VHL-Hypoxia-inducible factor (HIF)-Erythropoietin (EPO) pathway in RCC, we found HIF-2 α is expressed more frequently than HIF-1 α , and is more important in up-regulating the downstream molecules. Activation of EPO pathway is involved in cell growth, invasion, survival, and sensitivity to the targeted drug in RCCs, this is a potential therapeutic target for renal cancer.

Key words: erythropoietin, mutation, targeted therapy, von Hippel-Lindau (*VHL*) gene

Introduction

Renal cell carcinoma (RCC) accounts for approximately 3% of adult malignancies and represents 90–95% of neoplasms arising from the kidney^{1) 2)}. Renal cancer occurs in both sporadic and a hereditary form, and both forms are associated with structural alterations of the short arm of chromosome 3 (3p)³⁾. Von Hippel-Lindau (*VHL*) gene locates at chromosome 3p25–p26⁴⁾, a region of the genome that is frequently deleted or altered in RCC. It displays tumor suppressor effect through the gene product VHL protein to degrade the hypoxia induced factor (HIF) and inhibit the expression of

hypoxia response genes such as vascular endothelial growth factor (VEGF), erythropoietin (EPO), platelet derived growth factor (PDGF), etc., these products have been shown to be linked to the pathogenesis in the RCCs⁵⁾. These years, we have been doing a systematic research around the *VHL* gene pathway.

Characteristics of von Hippel-Lindau disease in Chinese patients

VHL disease (MIM 193300) is an autosomal dominant hereditary cancer syndrome caused by germline mutations in *VHL* gene, and this disease is

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Figure-1 Onset age in the parent-child pairs
Kaplan-Meier curve indicates the difference of onset age in 34 parent-child pairs (log-rank test, $p < 0.001$).
(Ning XH, *et al*: Cancer Res, 2014; 74: 3802-3809⁹⁾)

the most common hereditary renal cancer in the clinic. The incidence of VHL disease is estimated to be 1/36,000–52,000⁶⁾. This disease is characterized by a wide spectrum of tumors, including central nervous system hemangioblastoma, clear cell RCCs, retinal angioma, pancreatic cyst and tumor, pheochromocytoma, endolymphatic sac tumor, and papillary cystadenoma in epididymis or broad ligament⁶⁾.

As a rare disease, the data are insufficient to characterize the clinical and molecular findings of VHL disease in Chinese population. We used polymerase chain reaction (PCR)-direct sequencing and universal primer quantitative fluorescent multiplex PCR (UPQFM-PCR) method to detect point mutations, small and large indels in VHL in clinically diagnosed or suspected VHL disease patients. Our center has diagnosed hundreds of VHL patients in China, and based on their data, we have summarized the characteristics of them.

Of the 102 VHL disease families we recruited from various regions in China, 46 (45.1%) families had no family history, and 47.4% carried novel mutations. The prevalence of novel mutations without family history was higher in this group of patients, presumably demonstrating the higher prevalence of de novo mutations in VHL gene in Chinese⁷⁾.

We reported the case of a VHL mosaic patient

Figure-2 Relationship between age and relative telomere length in patients with VHL disease and normal controls

In normal controls ($n = 325$), the relative telomere length is negatively correlated with age, with the linear regression equation of $Y = 1.4503 - 0.0117 * X$, $R^2 = 0.162$ (the continuous line). In patients with VHL disease ($n = 29$), the average relative telomere length (the dotted line) is slightly lower than that in normal controls.

(Ning XH, *et al*: Cancer Res, 2014; 74: 3802-3809⁹⁾)

with bilateral renal lesions in the absence of other VHL-associated lesions. VHL mutation was not originally detected by routine molecular testing. Nonetheless, the detection of a heterozygous c.194C>G (p.Ser65Trp) VHL mutation in the patient's daughter prompted further genetic assessment and eventually resulted in the finding of a mosaic c.194C>G (p.Ser65Trp) VHL mutation in the patient. As the frequency of VHL mosaicism remains underdetermined, the possibility of a diagnosis of mosaic VHL should be considered in patients with both typical and atypical VHL-associated manifestations⁸⁾.

Anticipation is a phenomenon that the successive generations progressively manifest earlier onset age and more serious presentations for an inherited disease. We investigated anticipation in 18 Chinese VHL disease families. Onset age was younger in child than in parent in 31 of the 34 parent-child pairs (Figure-1). Patients in the first generation had older onset age with longer age-adjusted relative telomere length, and those in the next

generation had younger onset age with shorter age-adjusted relative telomere length ($p < 0.001$) in the 10 parent-child pairs from eight families with VHL disease. In addition, relative telomere length was shorter in the 29 patients with VHL disease than in the normal controls ($P = 0.003$) (Figure-2). The anticipation may relate to the shortening of telomere length by haplo-insufficiency of *VHL* in patients with VHL in successive generations. These findings indicate that anticipation is present in

families with VHL disease and may be helpful for genetic counseling for families with VHL disease families and for further understanding the pathogenesis of VHL disease⁹.

Research on VHL-HIF-EPO pathway

In our earlier studies, we found the frequency of *VHL* gene mutation in primary sporadic RCCs was about 53%, the incidence of death or metastasis of

Figure-3 Change of signaling molecules, proliferation, apoptosis, invasion ability and MMP-2 expression in 786-0 cells with knockdown of EPOR

A. Signaling molecules were assayed in 786-0 expressing EPOR siRNA (EPOR siRNA) by western blot, using parental 786-0 cells (control) and 786-0 expressing negative siRNA (Neg siRNA) as controls. In 786-0 cells expressing EPOR siRNA, EPOR and p-EPOR decreased by 90%, p-STAT5 decreased by 60%, but p-Erk1/2 increased by 36%, as compared with those in the two controls. B. Proliferation rate reduced significantly in 786-0 expressing EPOR siRNA by modified MTT method (experiments in triplicate; $p < 0.01$), as compared with that at the same time point in the two controls. C. Invasion ability decreased by about 55% in 786-0 expressing EPOR siRNA by transwell test (tests in triplicate $p < 0.001$). D. MMP-2 expression was inhibited by 33% in 786-0 expressing EPOR siRNA by gelatin zymography, and MMP-9 expression was very low in the 3 groups of 786-0 cells. Crystal violet stain shows different group of cells after transwell test. E. Early apoptotic cells (annexin V-PE+/7-AAD-) increased by about 4 times in 786-0 expressing EPOR siRNA, as compared to those of 786-0 expressing negative siRNA by flow cytometry (experiments in triplicate $p < 0.001$). (Wu P, et al: PLoS One, 2012; 7: e45122¹²)

Figure-4 Knockdown of EPOR suppresses 786-0 tumor growth in nude mice

Nude mice were subcutaneously inoculated with 2×10^6 parental 786-0 cells (Control, n=6), 786-0 cells stably expressing negative siRNA (Neg SiRNA, n=7), or 786-0 cells stably expressing EPOR siRNA (EPOR siRNA, n=10). A. Tumor sizes after the inoculation for 10 weeks, tumor is showed by red arrow (B) comparison of tumor size after animals were sacrificed. C. Changes of tumor size within 10 weeks (**: $p < 0.01$, ***: $p < 0.001$). (Wu P, *et al*: PLoS One, 2012; 7: e45122¹²⁾)

clear cell RCC in *VHL* gene mutation group is lower than non-mutation group. Loss of heterozygosity (LOH) of *VHL* gene is an important genetic event in Chinese sporadic RCC.

In RCCs, HIF-1 α , HIF-2 α and EPO expression are frequently observed in sporadic clear cell RCC. Although both HIF forms up-regulate expression of EPO, the relationship to HIF-2 α appears more pronounced. Initial results are thought-provoking and identify the VHL-HIF-EPO mechanism for further study¹⁰⁾.

Co-expression of EPO and erythropoietin receptor (EPOR) has been found in various non-hematopoietic cancers including hereditary and sporadic RCC¹¹⁾. We used RNA interference method to downregulate EPOR to investigate the function of EPO/EPOR pathway in human RCC cells. Down-regulation of EPOR expression in 786-0 cells by lentivirus-introduced siRNA resulted in inhibition

of growth and invasiveness *in vitro* and *in vivo*, and promotion of cell apoptosis (Figure-3, 4). In addition, rhEPO stimulation slightly antagonized the anti-tumor effect of Sunitinib on 786-0 cells. Sunitinib could induce more apoptotic cells in 786-0 cells with knockdown EPOR expression. Our results suggested that EPO/EPOR pathway was involved in cell growth, invasion, survival, and sensitivity to the multi-kinases inhibitor Sunitinib in RCC cells. And this pathway is a potential therapeutic target for renal cancer¹²⁾.

Conclusion

VHL gene is important to both hereditary and nonhereditary RCC, and VHL-HIF-EPO/EPOR pathway is involved in cell growth, invasion, survival, and sensitivity to the targeted drug in renal cell carcinoma. EPO/EPOR pathway is a

potential therapeutic target for renal cancer.

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