HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Original research http://dx.doi.org/10.1016/j.apjtm.2015.05.005

Netrin-1: The new tumor markers in renal clear cell carcinoma

Bai-Sheng Gong, Qiang Feng*

Uropoiesis Surgical Department, Sichuan Academy of Medical Sciences Sichuan Province People's Hospital, Chengdu 610072, China

ARTICLE INFO

Article history:
Received 15 Mar 2015
Received in revised form 20 Apr 2015
Accepted 15 May 2015
Available online 25 June 2015

Keywords:
Netrin-1
Renal clear cell carcinoma
Tumor marker

ABSTRACT

Objective: To explore the expression of Netrin-1 protein in human renal clear cell carcinoma (RCCC) and the relationships between Netrin-1, pathology and prognosis.
Methods: 72 cases of RCCC admitted in our hospital from 2008 June to 2009 June and their adjacent tissues were selected for study. They included 30 cases in stage Ⅰ–Ⅱ, 42 cases in stage Ⅲ–Ⅳ; 9 cases in grade Ⅰ, 9 cases in grade Ⅱ, 40 cases in grade Ⅲ and 14 cases in grade Ⅳ. All cases were followed up for more than 5 years. Survival analysis lines were made by Kaplan–Meier method and the difference between groups was tested by the Log-rank test. The expression of Netrin-1 protein was detected by immunohistochemistry staining and its clinical significance was analyzed.
Results: Renal clear cell carcinoma: 51 cases in high expression of Netrin-1 and 21 cases in low expression, normal tissues: 12 cases in high expression of Netrin-1 and 60 cases in low expression, the difference between the two groups is significant (χ^2 = 42.921, P < 0.01). The difference of the expression of Netrin-1in Fuhrman grade and AJCC clinical stage is significant (χ^2 = 8.000, χ^2 = 6.203; P < 0.05). The 5-year survival rate in low protein expression group and in high protein expression group was 79% (17/21) and 62% (32/51). The survival curve had different trend, with no significant difference between groups ((χ^2 = 1.360, P = 0.245).
Conclusions: Netrin-1 protein plays an important role in the development of RCCC. It might be a new specific tumor marker of RCCC, and might become a new target in treatment of RCCC.

1. Introduction

Renal clear cell carcinoma (RCCC), accounting for about 80% of renal malignant tumors, is one of the most common malignant tumor of the urinary system. The incidence of RCCC in China shows an increasing trend year by year [1]. It has changeable biological behavior, complex etiology and poor prognosis. It has become a current research hotspot to study the key factors of the occurrence and development of RCCC and how to improve the survival rate of the patients [2]. In recent years, many researchers concerned with the study on the correlation between the occurrence and development of tumor and Netrin-1. It has been found that Netrin-1 not only plays an important role in neural migration, neural guide and neural plasticity [3], but also can guide the growth of the glandular system and the vascular system, adjust the migration of white cell, exert a synergistic inhibition effect with the colorectal cancer homologous protein or the missing gene protein, or induce the apoptosis of tumor cell [4]. So Netrin-1 probably is a type of cancer gene and involved in the occurrence and development of tumor. In the study the Netrin-1 protein in RCCC tissues was detected by immunohistochemistry staining to explore its relationship with the occurrence and development of RCCC and its clinical significance.

2. Materials and methods

2.1. Clinical data

A total of 72 RCCC cases admitted in our hospital from 2008 June to 2009 June were selected for study, including 39 male cases and 33 female cases, ranging in age from 27 to 84 years old with a mean age of (56 ± 12) years old. Selection criteria of

*Corresponding author: Qiang Feng, Uropoiesis Surgical Department, Sichuan Academy of Medical Sciences Sichuan Province People's Hospital, Chengdu 610072, China.
E-mail: caotangfengqiang@126.com
Peer review under responsibility of Hainan Medical University.
Foundation project: It is supported by Sichuan Province Natural Science Foundation of China: sc271123.

patients was as following: ①The patients were at the first onset; ②The patients were diagnosed by routine pathological examinations; ③The patients were without biological treatment and radiotherapy and chemotherapy before the operation. The cancer tissues confirmed by pathological examinations removed in operation and their adjacent noncancerous tissues 2 cm beside the carcinoma were collected. The selected samples were embedded in paraffin and made into 4 μm sections. They included 30 cases in stage Ⅰ–Ⅱ, 42 cases in stage Ⅲ–Ⅳ; 9 cases in grade Ⅰ, 9 cases in grade Ⅱ, 40 cases in grade Ⅲ and 14 cases in grade Ⅳ.

2.2. Experimental reagents and methods

Immunohistochemical SP method was used. The primary antibody of Netrin-1, goat anti human polyclonal antibody, was bought from the British company Abcam. And the secondary antibody, rabbit anti goat SP kit, was bought from Fuzhou Maixin Biological Technology Development Company. The working concentration of the primary antibody was 1:100. The experimental procedure was carried out strictly in accordance with the instruction of the kit. Antigen repairing with citrate and DAB color was performed in paraffin sections after deparaffinating. The sections were stained with hematoxylin, dehydrated and sealed transparently. With the phosphate buffer solution as a negative control instead of the primary antibody and the known positive sections as a positive control, the sections were observed under the microscope [5].

2.3. Evaluation index

In the case of double-blind pathological and clinical data, the visual fields were selected by two pathologists and the ratio of positive staining cells and the total number of tumor cells was recorded. The results were determined separately and confirmed finally through discussion. Five high power fields (×400) were selected randomly in the samples of each patients, and the staining intensity and percentage of positive cells were selected as the reference index according to the related references [6]. Staining intensity was as follows: 0 score for no colouring, 1 score for pale yellow, 2 scores for brown yellow and 3 scores for brown; percentage of positive cells was as follows: 0 score for $\leq$10%, 1 score for 11%–25%, 2 scores for 26%–50% and 3 scores for $>$50%. The product of two scores was used as the basis of judgment. A sore of not less than 3 means high expression and a score of less than 3 means low expression.

2.4. Statistical analysis

All the data were processed using SPSS17.0 statistical software. According to the relationship between the clinical pathological parameters of RCCC and the expression level of Netrin-1 protein, a Fisher exact test or chi square test was used to analyze the difference between clinical data. The survival times were compared using Log-Rank (Mantel–Cox) test, and the survival rates were calculated using Kaplan–Meier method. A P value <0.05 was considered as statistically significantly different.

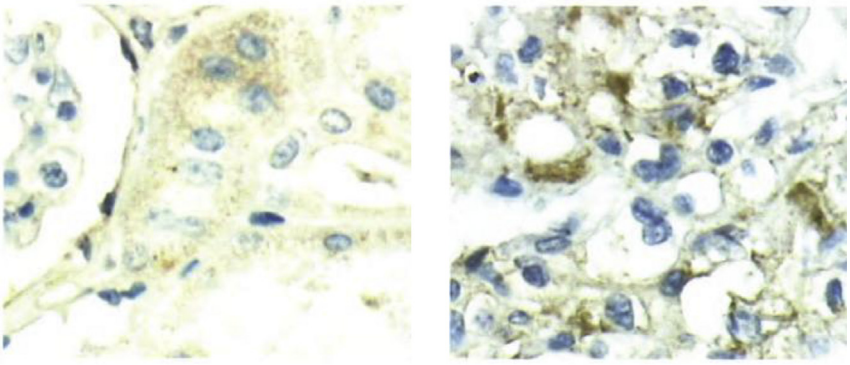
3. Results

3.1. Expression results of Netrin-1 in normal tissues adjacent to carcinoma and RCCC tissues

The Netrin-1 showed low expression in normal tissues adjacent to carcinoma and showed high expression in RCCC tissues with expression in the nucleus and cytoplasm (Figure 1). There were 51 cases in high expression of Netrin-1 and 21 cases

Table 1
Expression of Netrin-1 in 72 cases of RCCC.

Item	Number of cases	Expression of Netrin-1		χ^2 value	P value
		Low expression	High expression		
Gender					
Male	39	12	27	0.106	0.75
Female	33	9	24		
Age					
<60	39	13	26	1.309	0.253
$\geq$60	33	7	26		
Fuhrman grade					
Ⅰ–Ⅱ	18	9	9	8.000	<0.01
Ⅲ–Ⅳ	54	9	45		
AJCC clinical stage					
Ⅰ–Ⅱ	30	13	17	6.203	0.01
Ⅲ–Ⅳ	42	7	35		



A B

Figure 1. Expression of Netrin-1 in normal tissues adjacent to carcinoma and RCCC tissues.
A shows the low expression of Netrin-1 in normal tissues adjacent to carcinoma, and B shows the high expression of Netrin-1 in RCCC.

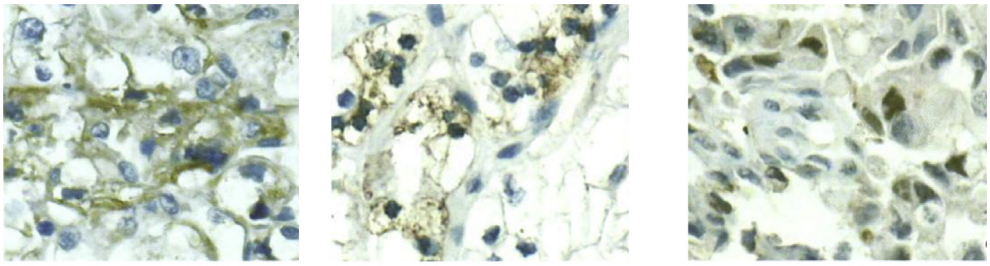


Figure 2. Expression of Netrin-1 protein in different pathological grade of RCCC tissues.
A: Fuhrman Ⅱ stage; B: Fuhrman Ⅲ stage; C: Fuhrman Ⅳ stage.

in low expression; 12 cases in high expression of Netrin-1 and 60 cases in low expression, the difference between the two groups was significant ($\chi^2 = 42.921$, $P < 0.01$).

3.2. Relationship between the clinical pathological features of RCCC and the positive expression of Netrin-1

The difference of the positive expression of Netrin-1 in 72 cases of RCCC in age and gender was not statistically significant ($P > 0.05$), while the difference of the expression of Netrin-1 in pathological grade and clinical stage was statistically significant ($P < 0.05$) (Table 1). In Fuhrman grade Ⅳ, Netrin-1 was also expressed within the nucleus in addition to the cytoplasm (Figure 2).

3.3. Analysis of the correlation between the postoperative survival rates of patients with RCCC and the expression of Netrin-1 protein

The 72 cases of RCCC had been followed up for more than 5 years by December, 2014. The 5-year survival rate after operation in low protein expression group and in high protein expression group was 79% (17/21) and 62% (32/51). Survival curves were made by Kaplan–Meier method and then the difference between groups was analyzed by the Log-rank (Mantel–Cox) test. There was no significant difference in survival rate between two group ($\chi^2 = 1.360$, $P = 0.245$) (Figure 3).

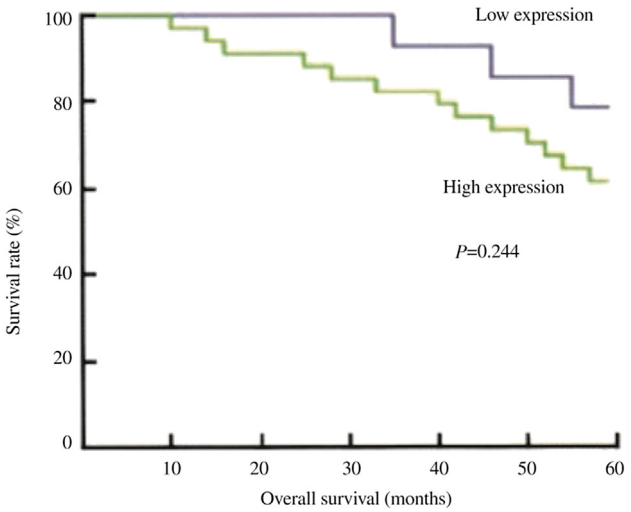


Figure 3. Survival curves after operation of patients with RCCC in low Netrin-1 expression group (21 cases) and in high Netrin-1 expression group (51 cases).

4. Discussion

4.1. Research status of Netrin-1 protein

Netrin-1 protein was found through the genetic analysis of UNC-6 deletion mutant of nematode. With a molecular weight of about 70–80 kD, Netrin-1 protein shows a high expression in the ventral area of ventricle and the bottom plate of spinal cord in development, and combines the location and growth of neurons by stimulating the growth of spinal cord [7]. As a homolog of UNC-6 in spinal animal, Netrin-1 protein is similar to laminin in structure. It is expressed in many parts of the body, such as the neuroglial cell in the ventral spinal cord, the internal capsule, the ventral area of lateral ventricle, and the cerebellar cortex in the nervous system; and the pancreas, the acinus of mammary gland, the myocardial cell, the dorsal part of artery and the ovary in the non nervous system [8]. Netrin-1 protein combines with transmembrane receptor like immunoglobulin to transfer signals through dependent receptor [9]. As one of the axon guidance factors in Netrin family, Netrin-1 protein is highly expressed in the nervous system, and involved in the axonal directional migration and growth as well as the neuronal development and differentiation. It plays a role of repulsion or attraction on axon according to different binding receptors in the development of central nervous system. Netrin-1 protein can direct cell migration and regulate axonal growth in the embryonic neural growth and development, and can also play roles of inducing cell migration, resisting apoptosis and regulating the formation of axonal direction when the adult central nervous system is damaged [10]. The nervous system is structurally similar to the vascular system, and the neuron and vessel are similar in growth pattern, so Netrin signal also play a role in the vascular system. It has been shown that Netrin-1 protein can promote the migration, proliferation and adhesion of endothelial cell, and the effect of VEGF. It has also been found to promote the formation of the artery as well as the micrangium [11]. The relationship between Netrin-1 and tumor formation becomes more and more important in our study. It has been found that the expression of Netrin-1 increases significantly in 47% patients with non small cell carcinoma [12], and it is also high in 7% patients with colon cancer [13]. The induced high expression of Netrin-1 in gastrointestinal tissues can reduce cell apoptosis and induce the occurrence of cancer. Netrin-1 protein influences the occurrence and development of gastric cancer by regulating the tumor cells, and helps evaluate the survival time and the

clinical pathological parameters of patients with gastric cancer. A variety of tumor tissues show high expression of Netrin-1 protein, such as metastatic breast cancer [14], neuroblastoma, non small cell lung cancer and pancreatic cancer [15]. And the current clinical treatment effect of these tumors is not ideal, needing more effective treatments. Ii is suggested that we need to have a deeper understanding of such molecules, and study its function in the occurrence and development of tumor.

4.2. Analysis of Netrin-1 protein expression

The expression of Netrin-1 in normal tissues adjacent to carcinoma and RCCC tissues was detected by immunohistochemistry staining in this study. There were 51 cases in high expression of Netrin-1 and 21 cases in low expression in RCCC, while there were 12 cases in high expression of Netrin-1 and 60 cases in low expression in normal tissues adjacent to carcinoma. The difference between the two groups is significant ($\chi^2 = 42.921$, $P < 0.01$), indicating that the positive expression of Netrin-1 protein RCCC is higher than that in normal renal tissues adjacent to carcinoma; the difference of the positive expression of Netrin-1 in 72 cases of RCCC in pathological grade and clinical stage is statistically significant ($P < 0.05$), showing that the expression of Netrin-1 in RCCC increases with the increasing pathological grade and clinical stage, which is agreed by Li *et al* [16]. It is speculated to be related with the participation of Netrin-1 in the expression regulation mechanism in tumor [17]: UNC5B is a downstream gene of p53 gene, containing a p53 binding sequence in its first intron. UNC5B can be regulated down through RNA interference to inhibit the apoptosis induced by p53 significantly, indicating that UNC5B is an important target of the transcription level of p53 induced cell apoptosis, and can play a role of promoting apoptosis. In the case of p53 mutation or deletion in tumor, the expression of UNC5B decreases significantly, and Netrin-1 can inhibit the apoptosis induced by p53 completely. When UNC5B combines with Netrin-1, although p53 protein can still stably be expressed and gather, it is in the inactivated state on the function. Therefore, Netrin-1 can be considered a proto-oncogene. It has been found that NF-κB is in the activated state in the production of tumor, making the expression of a variety of genes increasing [18]. There is a conserved binding sequence of NF-κB within the startup regulating zone of Netrin-1 through DNA horizontal comparison. It can be proved by the detection of ChIP and genes that NF-κB can upregulate the expression of Netrin-1, thus inducing cell migration and survival [19].

4.3. Analysis of the correlation between the postoperative survival rates of patients with RCCC and the expression of Netrin-1 protein

It is shown in the study that the 5-year survival rate after operation in low Netrin-1 protein expression group and in high Netrin-1 protein expression group was 79% (17/21) and 62% (32/51), with no significant difference in survival rate between two group ($\chi^2 = 1.360$, $P = 0.245$). Although there is no significant difference between groups, we can find that the survival rate in high expression group is lower than that in low expression group, with an obvious trend. It is inferred to be related to the limited samples in this study. The next step can be improving the study sample size to explore the relationship between the expression of Netrin-1 and the survival rates of patients more accurately [20].

In conclusion, the study results show that the expression of Netrin-1 is significantly correlated to the pathological grade and clinical stage of RCCC, and may be related with the survival rate of patients. Netrin-1 may become a new biomarker in the prognosis and diagnosis of RCCC. Through further study on the specific mechanism of Netrin-1 in the occurrence and development of RCCC, Netrin-1 is expected to improve a new strategy for the diagnosis and treatment of renal carcinoma.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Mazzanti CM, Tomei S, Di Cristofano C, Minervini A. VHL and HIF-1α: gene variations and prognosis in early-stage clear cell renal cell carcinoma. *Med Oncol* 2014; **209**(3): 1-7.
[2] Lin L, Zhong K, Sun Z. Receptor for advanced glycation end products (RAGE) partially mediates HMGB1-ERKs activation in clear cell renal cell carcinoma. *J Cancer Res Clin* 2012; **138**(5): 11-22.
[3] Kruck S, Merseburger AS, Hennenlotter J, Scharpf M, Eyrich C, Amend B, et al. High cytoplasmic expression of p27 Kip1 is associated with a worse cancer-specific survival in clear cell renal cell carcinoma. *Bju Int* 2012; **109**(2): 1565-1570.
[4] Fergelot P, Bernhard J, Soulet F, Kilarski WW, Léon C, Courtois N, et al. [The experimental renal cell carcinoma model in the chick embryo]. *Angiogenesis* 2013; **312**(16): 181-194.
[5] Lu D, Dong D, Zhou Y, Lu M, Pang XW, Li Y, et al. The tumor-suppressive function of UNC5D and its repressed expression in renal cell carcinoma. *Clin Cancer Res* 2013; **212**(11): 2883-2892.
[6] Han M, Li Y, Liu M, Cong B. Renal neutrophil gelatinase associated lipocalin expression in lipopolysaccharide-induced acute kidney injury in the rat. *BMC Nephrol* 2012; **13**(4): 387-397.
[7] Ma WJ, Zhou Y, Lu D, Dong D, Tian XJ, Wen JX, et al. Reduced expression of Slit2 in renal cell carcinoma. *Med Oncol* 2014; **231**(1): 1-7.
[8] Baudet M, Bellon A, Holt CE. Role of microRNAs in Semaphorin function and neural circuit formation. *Semin Cell Dev Biol* 2013; **412**(24): 146-155.
[9] Shimizu A, Hirono S, Tani M, Okada KI, Miyazawa M, Shimizu A, et al. Coexpression of MUC16 and mesothelin is related to the invasion process in pancreatic ductal adenocarcinoma. *Cancer Sci* 2012; **103**(6): 739-746.
[10] Kaur P, Mani S, Cros MP, Scoazec JY, Chemin I, Hainaut P, et al. Epigenetic silencing of sFRP1 activates the canonical Wnt pathway and contributes to increased cell growth and proliferation in hepatocellular carcinoma. *Tumor Biol* 2012; **176**(33): 325-336.
[11] Bell JL, Wächter K, Mühleck B, Pazaitis N, Köhn M, Lederer M, et al. Insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs): post-transcriptional drivers of cancer progression? *Cell Mol Life Sci* 2013; **198**(70): 2657-2675.
[12] Zhan B, Kong C, Guo K, Zhang Z. PKCα is involved in the progression of kidney carcinoma through regulating netrin-1/UNC5B signaling pathway. *Tumor Biol* 2013; **34**(3): 1759-1766.
[13] Coffey RJ. LRIG1 is a triple threat: ERBB negative regulator, intestinal stem cell marker and tumour suppressor. *Brit J Cancer* 2013; **47**(5): 272-277.
[14] Ranganathan P, Jayakumar C, Santhakumar M, Ramesh G. Netrin-1 regulates colon-kidney cross talk through suppression of IL-6 function in a mouse model of DSS-colitis. *Am J Physiol Renal* 2013; **239**(9): F1187-F1197.

[15] Yan W, Han P, Zhou Z. Netrin-1 induces epithelial–mesenchymal transition and promotes hepatocellular carcinoma invasiveness. *Dig Dis Sci* 2014; **31**(12): 2499-2512.
[16] Li Q, Shi R, Wang Y, Niu X. TAGLN suppresses proliferation and invasion, and induces apoptosis of colorectal carcinoma cells. *Tumor Biol* 2013; **34**(1): 505-513.
[17] Tu Y, Wang H, Sun R, Ni Y, Ma L, Xv F, et al. Urinary netrin-1 and KIM-1 as early biomarkers for septic acute kidney injury. *Ren Fail* 2014; **184**(36): 1559-1563.
[18] Wang H, Zhang B, Gu M, Li S, Chi Z, Hao L. Overexpression of the dependence receptor UNC5H4 inhibits cell migration and invasion, and triggers apoptosis in neuroblastoma cell. *Tumor Biol* 2014; **35**(6): 5417-5425.
[19] Harter PN, Zinke J, Scholz A, Tichy J, Zachskorn C, Kvasnicka HM, et al. Netrin-1 expression is an independent prognostic factor for poor patient survival in brain metastases. *Plos One* 2014; **146**(3): e92311.
[20] Yang G. Netrin-1 overexpression promotes white matter repairing and remodeling after focal cerebral ischemia in mice. *J Cerebr Blood F Met* 2013; **33**(2): 1921-1927.