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Twist2 and CD24 expression alters renal microenvironment in obesity associated kidney cancer

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Abstract. – OBJECTIVE: Obesity emerged as a major public health problem worldwide, and prolonged condition with increased BMI causes various metabolic disorders include the development of kidney cancer. The metabolic changes alter the renal microenvironment and thereby promoting tumor. Hence, detailed studies of genes that regulate these this changes are keen to understand.

MATERIALS AND METHODS: Initially, we successfully initiate kidney tumor using prolonged intake of a high-fat diet in Wistar rats, which are confirmed with pathological changes observed through histological sectioning. The expression of Twist2 and CD24 was assessed using Immunohistology and Western Blotting in a different time interval of kidney cancer.

RESULTS: The rats fed with high-fat diet for 8 months shows 1.5 times increased in body mass whereas rats fed with high-fat diet for 16 months shows triple the size when compared with controls. Histological sectioning confirms the development of lesions and proteinaceous casts in 8 months high-fat fed rats, whereas we observed the high proliferative mass of cells in 16 months high-fat fed rats. Interestingly, we also observed elevated expression of Twist2 in initial stages of kidney cancer, which are down-regulated in the latter stages of kidney cancer. The experiments with CD24 shows the gradual increase of the expression of CD24 as a tumor develops to the next level.

CONCLUSIONS: The correlation between Twist2 and CD24 expression conclude that Twist2 overexpression in initial stage augments CD24 to express more in the latter stage of kidney cancer. Reversely, the overexpression of CD24 and down-regulation of Twist2 in later stages of kidney cancer suggest the CD24 expression is dependent on Twist2 expression level.

Key Words:
Kidney cancer, Obesity, Twist2, CD24, High-fat diet.

Introduction

Kidney cancer accounts for nearly 270,000 new cases and ends with the death of 115,000 cases in each year[1]. In 20-30% of cancer patients, it appears in the form of metastasis and is primarily treated with surgery[2]. The major reason behind the development of renal carcinoma is obesity, smoking, and hypertension[3]. Among them, hypertension is found to be increasing the risk factors in many renal carcinoma cases[4]. Patients with obesity usually developed with hypertension and they together shared a common mechanism associated with renal cell carcinoma[5]. Since the renal carcinoma is incurable, preventive measures are ideal for this disease by changing lifestyle risk factors.

Obesity is a primary risk factor for renal carcinoma as it alters renal microenvironment by increasing glomerular filtration and renal plasma flow, which ultimately results in renal damage and carcinoma[6,7]. Obesity and hypertension in together increase oxidative stress and lipid peroxidation[8,9] which thereby damage the renal cells. Treatment resistance is another problem associated with renal carcinoma as it shows less responsive to chemotherapy, as well as radiotherapy, and, for those patients, radical nephrectomy is the last choice[10]. Other than that recurrence of cancer in local or distant tissue makes difficult to control due to poor prognosis. Therefore, understanding the molecular mechanism from primary lesions to metastasis is crucial for early prognosis and treatment[11].

Twist2 gene codes for a transcriptional factor that control cell differentiation in many cell types during early development of embryogenesis[12]. Recent studies show that some signaling pathways like Notch, Wnt and Twist play a role in early development also regulate the development of a various type of cancer in latter stages[13,14]. Twist2 is

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overexpressed in many cancer tissues and likely involved in tumor progression by disseminating the cancer cells[15]. CD24 is a renal progenitor marker involved in the reparative process following injury or disease condition[16]. In this article, we try to understand the role of Twist2 and CD24 in different pathological stages of obesity-associated renal cancer.

Materials and Methods

Animals

For inducing the renal tumor, Female Wistar rats (n=24; age 3 months; 200-250 g) were chosen because they are very sensitive to the diet-induced renal tumor when compared with male rats. Of the total rats, eight (n=8) are provided with a regular chow diet (15% calories from fat, 60% from carbohydrates), and the remaining rat is fed with a high-fat diet (42% calories from butterfat, 44% from corn starch) ad libitum. One group of control (n=4) and high-feed rat (n=8) are sacrificed at the end of 8 months by decapitation. For the remaining control and high-fat rat groups the feed was continued until 16 months, and later they were sacrificed. All the experimental animals are obtained from the animal house maintained in the Institution and approved by the Ethical Committee of the host institution.

Histopathological Changes

The rats were dissected to remove both left and right kidney. The renal samples from control and high-fat diet rats in different time point were subjected to histology and stained with H&E. Initially, the kidney samples were excised and fixed in 10% formalin solution. After gradual dehydration with ethanol, the sections are treated with xylene that acts as a clearing agent. Following that the tissue is embedded in paraffin wax and dissected using microtome (6µm size). The sections are processed and finally stained with hematoxylin and eosin.

Immunohistochemistry

The paraffin-embedded kidney samples were subjected to thin sectioning using microtome and latter deparaffinized by heating in slide warming table and by sudden transfer to xylene solution. The slides are then dipped in an alcohol solution and were rehydrated. The sections are then treated with 5% H_2O_2 in methanol to block the activity of endogenous peroxidase activity. To unmask the antigen, the sections are treated with 0.1% trypsin solution in 0.1% $CaCl_2$ for 5 minutes at room temperature. Following washing with 1X PBS, the sections are incubated with primary antibody Twist2 or CD24 at 4º C for 6 hours. The antibody that binds non-specifically to the section is washed away with 1X PBS and incubated with a secondary antibody that is specific to FC region of a primary antibody. Following the immunohistological protocol, the antigen-antibody complex is visualized using color reaction using diaminobenzidine (DAB) as a chromogen.

Western Blotting

The expression of specific protein Twist2 or CD24 were detected using western blotting. The kidney samples dissected from control and diet-induced rats were homogenized in 2X protein sample buffer, and protein samples were prepared. The protein samples from different kidney tissues are loaded in a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate the proteins based on the mass/charge ratio. The separated proteins are transferred to a membrane and incubated with primary antibody for Twist2 or CD24. The detection of the primary antibody was carried out using HRP-conjugated secondary antibody.

Results

Developing Diet-Induced Kidney Cancer

To develop an effective diet-induced kidney cancer, we fed the rats with high-fat diet as described in materials and method section. One group of rats were fed for 8 months, and another group of rats the diet was continued for another 8 months. The controls are fed with regular chow diet as mentioned already. We observed when compared with the control rats (350 ± 50 g) the weight of 8 months high-fat diet fed rats showed 1.5 times more weight (530 ± 30 g). But the weight of rats fed with 16 months of high-fat diet fed shows significant weight gain (1300 ± 45 g) which is triple the size when compared with control mice, which are fed with a normal diet for 16 months (400 ± 20 g). The result shows obesity burden which has the ability to change the normal physiology of the rats and possibly may develop with renal carcinoma[17].

Changes in Renal Microenvironment

The rat kidney which shows excessive tumor growth are dissected and subjected to histological

analysis. The histological sections of control rat kidney show well organized cellular structures with a uniform gap between the two cells and also it was observed without any lesions (Figure 1A). In contrast, the rat that is fed with high-fat diet for 8 months develops renal lesions along with proteinaceous casts and thickened extracellular layers (Figure 1B). A rat that is fed with high-fat diet for another 8 months shows more proliferative cells with high lesions (Figure 1D). But we observed cancerous condition only in 3 rats out of 8 rats when compared with their respective controls (Figure 1C).

Understanding the Expression of Twist2 in Different Pathological Stages of kidney Cancer

Twist2 plays a major role in tumorigenesis and are involved in cell proliferation, suppressing apoptosis, enhance cellular invasion and its migration[18]. To evaluate the expression of Twist2 in different critical stages of kidney cancer the kidney samples isolated from control, rats fed with high-fat diet for 8 and 16 months are subjected to immunohistological analysis. The control kidney tissue shows limited expression in dispersed cells (Figure 2A),

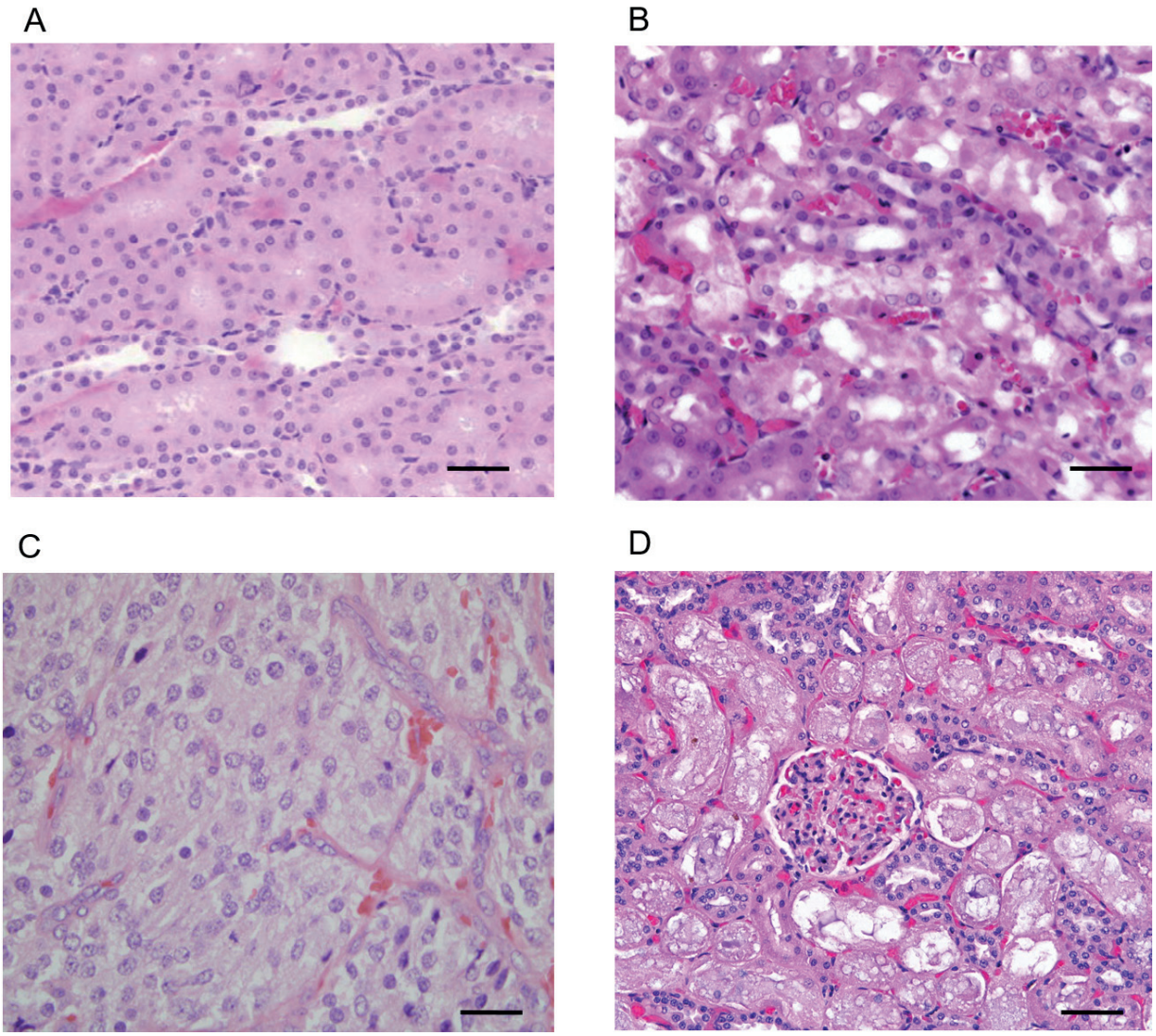

Figure 1. Histological of diet-induced kidney cancer. ***A,*** Control kidney tissue without any lesions. ***B,*** Early kidney cancer showed lesions with proteinaceous casts and thickened basal membrane. ***C,*** Prolonged intake of high-fat diet results with more proliferative cells. Scale = 50 μm size.

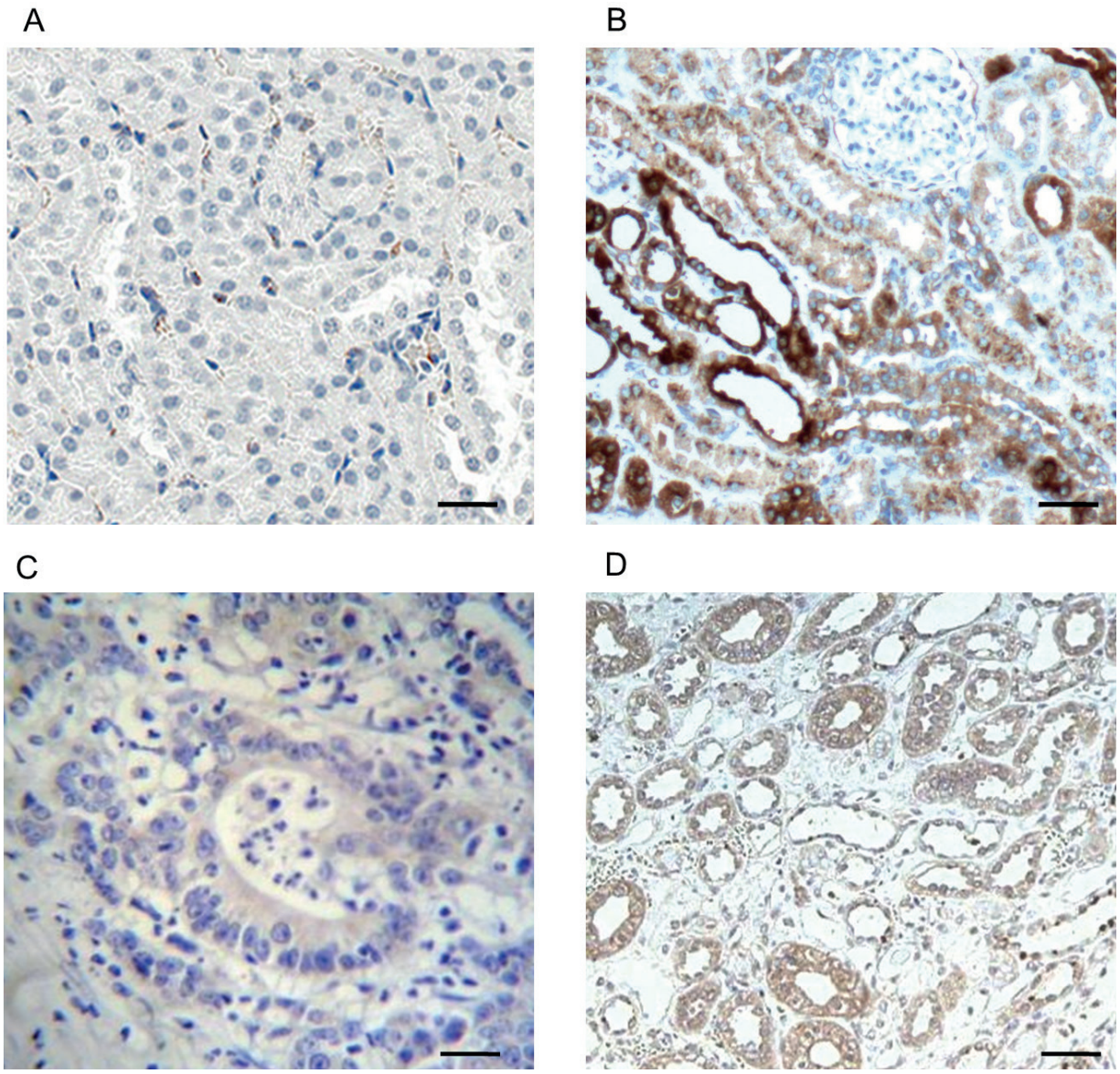

Figure 2. Immunohistochemistry analysis of Twist2 expression. ***A,*** Weak expression of Twist2 in control kidney tissue of 8 months normal fed rat. ***B,*** Over expression of Twist2 in the early stage of kidney cancer. ***C,*** Weak expression of Twist2 in control kidney tissue of 16 months normal fed rat. ***D,*** Significant down regulated expression of Twist in an advanced stage of kidney cancer. Scale = 50 μm size.

but subsequently, their expression significantly overexpressed in rats fed with high-fat diet for 8 months (Figure 2B). However, in rats fed with high-fat diet for 16 months shows a reduction in their expression level (Figure 2D) when validating with their control tissue (Figure 2C).

The link Between CD24 Expression and Tumor Progression

CD24 is a GPI-linked membrane glycoprotein that is associated with tumor progression in many types of cancer[19], and it is also involved in promoting metastasis[20]. The expression of CD24 in normal kidney tissue reflects the minimal expression as shown in Figure 3A. But its expression shows limited upregulation in a small subpopulation of cells in rats fed with high-fat diet for 8 months (Figure 3B). However, we observed the higher expression of CD24 as the proliferative cells increased as in the case of rats fed with high-fat diet for 16 months (Figure 3D) when compared with their control (Figure 3C).

Western Blotting Analysis

The results demonstrated using immunohistochemistry are validated together with Western blotting. The expression of two proteins, Twist2 and CD24 shows a similar pattern of expression as analyzed using immunohistochemistry. The signal intensity of Twist2 shows overexpression pattern in rats fed with high-fat diet for 8 months when compared it with 16 months (Figure 4). As shown in Figure 3A-D, the expression pattern of CD24 shows gradual overexpression as tumor proceeds to next level (Figure 4).

Discussion

Obesity results with more complication, and it is associated with co-morbidities like cardiovascular disease, diabetes mellitus, Alzheimer's disease, hepatic steatosis, hypertension that leads to chronic kidney disease as well as results with renal cancer in certain cases[21-23]. Till the mechanism behind the obesity-associated kidney cancer remains unclear and detecting them in earlier may save many lives in the future. In the present studies, we analyzed the expression of Twist2 and

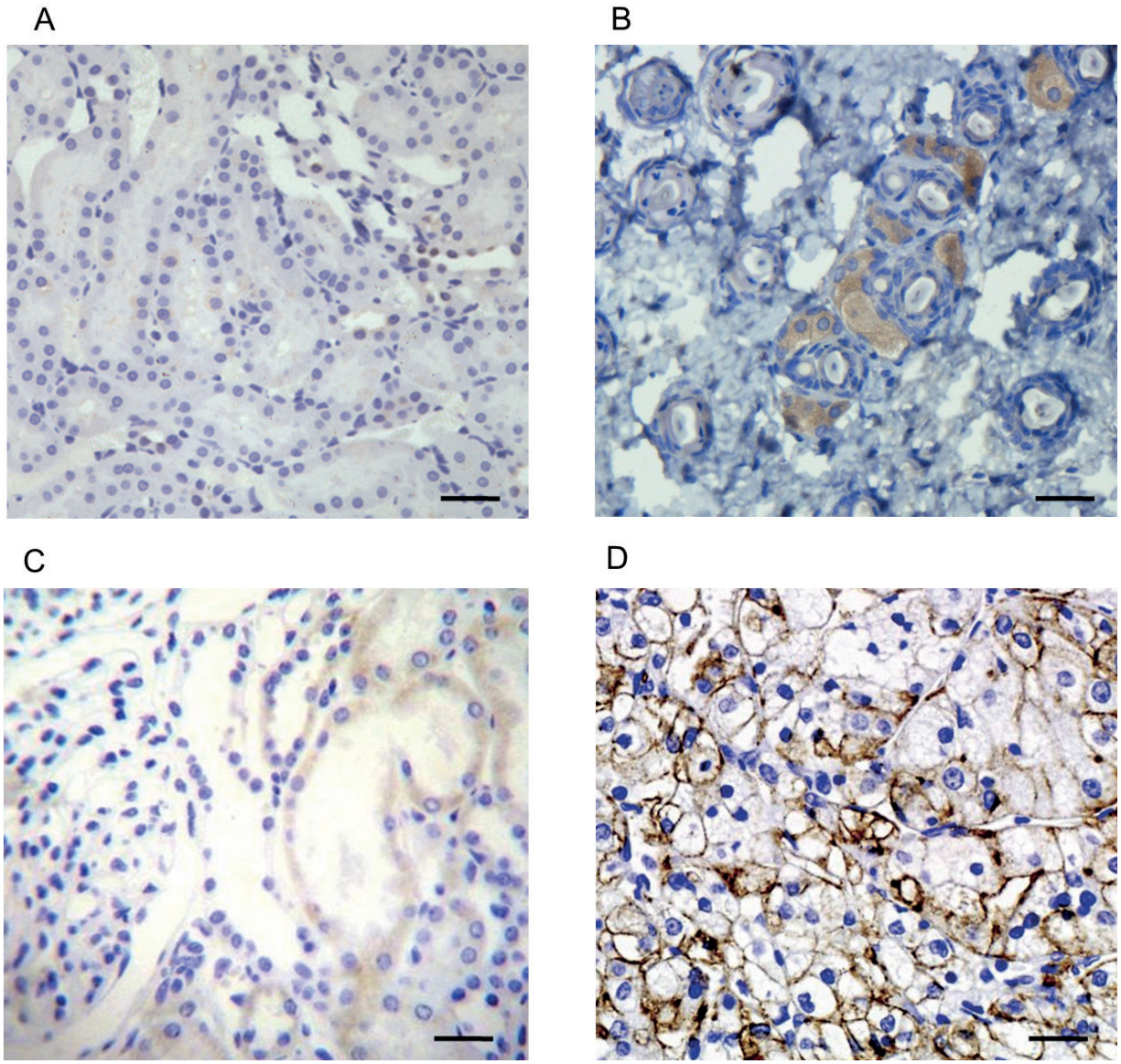

Figure 3. Immunohistochemistry analysis of CD24 expression. ***A,*** Weak expression of CD24 in control kidney tissue of 8 months normal fed rat. ***B,*** Moderate expression of CD24in early stage of kidney cancer. ***C,*** Weak expression of CD24 in control kidney tissue of 16 months normal fed rat. ***D,*** Over expression of CD24 in an advanced stage of kidney cancer. Scale = 50 μm size.

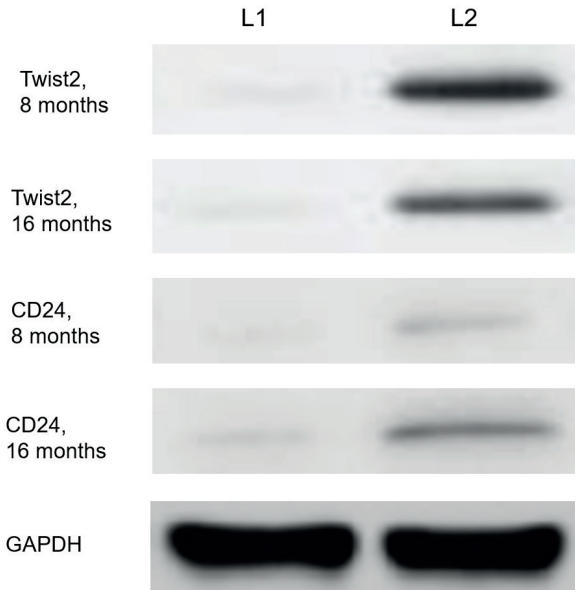

Figure 4. Western Blotting against anti-Twist2 and anti-CD24 protein expression. Overexpression of Twist2 was observed in initial kidney cancer, and it shows down-regulated expression in advance stage of kidney cancer. CD24 shows a gradual increase in their expression pattern as tumor proceed to the next level. L1 – control kidney; L2 – kidney cancer tissue. GAPDH was used as a loading control.

CD24 expression in different pathological stages of high-fat diet induced kidney cancer rat model.

The renal micro environmental changes that induce renal cancer is an interesting topic to investigate, and it helps to understand the basics behind obesity-associated kidney cancer. Our results define as the duration of high-fat diet continuous the developed lesions responded well and resulted in more proliferative cells (Figure 1A-D). In our studies, few rats only acquired renal cancer and the reasons why not all rats develop renal cancer may be due to individual response and epigenetic variability.

Our investigation with Twist2 shows that its expression is elevated in initial stages of kidney cancer, but it remarkably shows decreased expression in later stages of kidney cancer as in the case of rats fed with high-fat diet for 16 months (Figure 2A-D). The results obtained correlate with the recent findings that its expression is upregulated in early initial stages of acute kidney injury[24]. The precise mechanism of Twist2 overexpression in early stages of kidney cancer is are needed to understand which may aid in using it as an early marker to detect kidney cancer.

Recent studies highlight the importance of CD24 expression and their link with cancer stem cells[25,26]. There are also reports available that Twist2 expression regulates CD24 in liver cancer[27]. Our results also support this data that Twist2 overexpression in early kidney cancer may be due to the induction of CD24 expression level, which is low at this time (Figure 3B) and after CD24 overexpression in later stages of cancer (Figure 3D) the expression of Twist2 comes down.

Conclusions

We observed more proliferative renal cells as high-fat diet is continuing for a prolonged time. The expression of Twist2 shows varied expression pattern as it is more elevated in initial kidney cancer than in advanced stages which may drive CD24 expression.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References

1) Ferlay J, Shin H, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010; 127: 2893-2917.
2) Cohen HT, McGovern FJ. Renal-cell carcinoma. N Engl J Med 2005; 353: 2477-2490.
3) Tseng C-H. Type 2 diabetes mellitus and kidney cancer risk: a retrospective cohort analysis of the National Health Insurance. PLoS One 2015; 10: e0142480.
4) Weikert S, Boeing H, Pischon T, Weikert C, Olsen A, Tjonneland A, Overvad K, Becker N, Linseisen J, Trichopoulou A, Mountokalakis T, Trichopoulos D, Sieri S, Palli D, Vineis P, Panico S, Peeters PH, Bueno-de-Mesquita HB, Verschuren WM, Ljungberg B, Hallmans G, Berglund G, González CA, Dorronsoro M, Barricarte A, Tormo MJ, Allen N, Roddam A, Bingham S, Khaw KT, Rinaldi S, Ferrari P, Norat T, Riboli E. Blood pressure and risk of renal cell carcinoma in the European prospective investigation into cancer and nutrition. Am J Epidemiol 2007; 167: 438-446.
5) Setiawan VW, Stram DO, Nomura AMY, Kolonel LN, Henderson BE. Risk factors for renal cell cancer: the multiethnic cohort. Am J Epidemiol 2007; 166: 932-940.
6) Lipworth L, Tarone RE, McLaughlin JK. The epidemiology of renal cell carcinoma. J Urol 2006; 176: 2353-2358.
7) Khandekar MJ, Cohen P, Spiegelman BM. Molecular mechanisms of cancer development in obesity. Nat Rev Cancer 2011; 11: 886-895.

8) Gago-Dominguez M, Castelao JE, Yuan J-M, Ross RK, Mimi CY. Lipid peroxidation: a novel and unifying concept of the etiology of renal cell carcinoma (United States). Cancer Causes Control. 2002; 13: 287-293.

9) Gago-Dominguez M, Castelao JE. Lipid peroxidation and renal cell carcinoma: further supportive evidence and new mechanistic insights. Free Radic Biol Med 2006; 40: 721-733.

10) Harada K, Miyake H, Kusuda Y, Fujisawa M. Expression of epithelial-mesenchymal transition markers in renal cell carcinoma: impact on prognostic outcomes in patients undergoing radical nephrectomy. BJU Int 2012; 110: 956-960.

11) Ohba K, Miyata Y, Matsuo T, Asai A, Mitsunari K, Shida Y, Kanda S, Sakai H. High expression of Twist is associated with tumor aggressiveness and poor prognosis in patients with renal cell carcinoma. Int J Clin Exp Pathol 2014; 7: 3158-3165.

12) Franco HL, Casasnovas J, Rodríguez-Medina JR, Cadilla CL. Redundant or separate entities?—roles of Twist1 and Twist2 as molecular switches during gene transcription. Nucleic Acids Res 2010; 39: 1177-1186.

13) Wright TM, Brannon AR, Gordan JD, Mikels AJ, Mitchell C, Chen S, Espinosa I, van de Rijn M, Pruthi R, Wallen E, Edwards L, Nusse R, Rathmell WK. Ror2, a developmentally regulated kinase, promotes tumor growth potential in renal cell carcinoma. Oncogene 2009; 28: 2513-2523.

14) Nakagawara A. Trk receptor tyrosine kinases: a bridge between cancer and neural development. Cancer Lett 2001; 169: 107-114.

15) Ansieau S, Bastid J, Doreau A, Morel A-P, Bouchet BP, Thomas C, Fauvet F, Puisieux I, Doglioni C, Piccinin S, Maestro R, Voeltzel T, Selmi A, Valsesia-Wittmann S, Caron de Fromentel C, Puisieux A. Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence. Cancer Cell 2008; 14: 79-89.

16) Romagnani P, Remuzzi G. CD133+ renal stem cells always co-express CD24 in adult human kidney tissue. Stem Cell Res 2014; 12: 828-829.

17) Stemmer K, Perez-Tilve D, Ananthakrishnan G, Bort A, Seeley RJ, Tschöp MH, Dietrich DR, Pfluger PT. High-fat-diet-induced obesity causes an inflammatory and tumor-promoting microenvironment in the rat kidney. Dis Model Mech 2012; 5: 627-635.

18) Zhang H, Tao J, Sheng L, Hu X, Rong R, Xu M, Zhu T. Twist2 promotes kidney cancer cell proliferation and invasion by regulating ITGA6 and CD44 expression in the ECM-receptor interaction pathway. Onco Targets Ther 2016; 9: 1801-1812.

19) Kristiansen G, Sammar M, Altevogt P. Tumour biological aspects of CD24, a mucin-like adhesion molecule. J Mol Histol 2004; 35: 255-262.

20) Baumann P, Cremers N, Kroese F, Orend G, Chiquet-Ehrismann R, Uede T, Yagita H, Sleeman JP. CD24 expression causes the acquisition of multiple cellular properties associated with tumor growth and metastasis. Cancer Res 2005; 65: 10783-10793.

21) Haslam DW, James WP. Obesity. Lancet 2005; 366: 1197-1209.

22) Brunt EM. Pathology of nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol 2010; 7: 195-203.

23) Barnes DE, Yaffe K. The projected effect of risk factor reduction on Alzheimer's disease prevalence. Lancet Neurol 2011; 10: 819-828.

24) Grunz-Borgmann EA, Nichols LA, Wang X, Parrish AR. Twist2 is upregulated in early stages of repair following acute kidney injury. Int J Mol Sci 2017; 18: 368-375.

25) Yeung TM, Gandhi SC, Wilding JL, Muschel R, Bodmer WF. Cancer stem cells from colorectal cancer-derived cell lines. Proc Natl Acad Sci 2010; 107: 3722-3727.

26) Lee TKW, Castilho A, Cheung VCH, Tang KH, Ma S, Ng IOL. CD24+ liver tumor-initiating cells drive self-renewal and tumor initiation through STAT3-mediated NANOG regulation. Cell Stem Cell 2011; 9: 50-63.

27) Liu AY, Cai Y, Mao Y, Lin Y, Zheng H, Wu T, Huang Y, Fang X, Lin S, Feng Q, Huang Z, Yang T, Luo Q, Ouyang G. Twist2 promotes self-renewal of liver cancer stem-like cells by regulating CD24. Carcinogenesis 2013; 35: 537-545.