Canonical Wnt Signaling Inhibition in Renal Cell Carcinoma Bone Metastasis: Immunohistochemical Study of DKK1 and LRP5 Expression

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Research

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Abstract

Introduction & Objectives: Canonical Wnt signaling (Wnt/ β -catenin signaling) maintains the bone homeostasis by promoting the osteoblastic activities. The inhibitory factor, Dickkopf (DKK)1, enhances the bone resorption, especially in malignancies. The low density lipoprotein related protein (LRP) 5 is a component of membranous co-receptor of Wnt/ β -catenin signaling and is also involved in serum low density lipoprotein cholesterol (LDL-C) level regulation. The clear cell renal cell carcinoma bone metastasis (ccRCC-BM) is characterized by osteolytic bone resorption. Whether and how Wnt/ β -catenin signaling plays roles in regulating the invasion, metastasis and osteolytic process of ccRCC to bone remain unclear. This study investigated the expression of DKK1, LRP5 proteins in primary and metastatic lesions of RCC-BM. The therapeutic potential of Wnt/ β -catenin signaling target medication was also evaluated.

Materials & Methods: ccRCC-BM patients with paired samples of primary and metastatic lesions were selected. ccRCC patients without any metastasis (ccRCC-only) were set as control. Slides of paraffin-embedded tissue underwent immunohistochemical staining with monoclonal

anti-DKK1 antibody and polyclonal anti-LRP5 antibody. Semi-quantitatively scoring according to staining intensity was performed. The staining results in the renal tissue adjacent to RCC, the primary RCC lesions (with BM or without BM), and the RCC-BM lesions were recorded. The expression difference was analyzed by univariate analysis of variance (ANOVA).

Results: The expression of DKK1 was significantly different amid renal tissue adjacent to RCC, primary RCC and RCC-BM tissues ($p < 0.001$). The expression of DKK1 in primary RCC was significantly lower than that in renal tissue adjacent to RCC ($p < 0.001$). No difference was found between ccRCC-BM group and ccRCC-only group. DKK1 expression in bone metastasis was significantly higher than that in primary tumor ($p < 0.001$). The expression of LRP5 in the primary tumor of ccRCC-BM group was significantly lower than that of adjacent renal tissue ($p < 0.01$). Tendency of decreasing expression was found between primary lesion of ccRCC-BM group and primary lesion of ccRCC-only group ($p = 0.073$). In bone metastasis, the expression of LRP5 protein was not significantly different from that in adjacent renal tissue and RCC primary lesion.

Conclusions: A "rebound" of DKK1 expression was found in bone metastasis lesions. Along with the decreasing LRP5 expression in primary lesions of RCC-BM patients, this suggests that the canonical Wnt signaling (Wnt/ β -catenin signaling) is inhibited during the bone metastasis process in ccRCC. The overexpression of DKK1 and the down-regulation of LRP5 receptor are involved.

Introduction

Being an occasionally encountered situation, bone metastasis (BM) in renal cell carcinoma (RCC) patients haunted urological health care providers [1]. Bone pain, pathological fracture, nerve compression and consequent paraplegia greatly impair patients' quality of life and also reduce their survival time [2]. The recent advocated comprehensive therapeutic model, including resection of primary and metastasis

lesions, radiotherapy and systematic medications, still could not benefit all bone metastasis patients [3]. Among those medications, tyrosine kinase inhibitors (TKIs), targeting several growth factors and receptors, prolong the survival time to a certain extent. But disease progression with increasing number of lesions and symptoms aggravation is hard to avoid [4, 5]. This suggests that invasion and metastasis of renal cell carcinoma, especially to bone, might be regulated by other signalings.

Canonical Wnt signaling (Wnt/ β -catenin signaling) maintains the bone homeostasis by promoting the osteoblastic activities [6–8]. The inhibitory factor, Dickkopf (DKK)1, enhances the bone resorption, especially in malignancies related to osteolytic destruction [9–11]. The low density lipoprotein related protein (LRP) 5 is a component of membranous co-receptor of Wnt/ β -catenin signaling and is also involved in serum low density lipoprotein cholesterol (LDL-C) level regulation [12–14]. The clear cell renal cell carcinoma bone metastasis (ccRCC-BM) is characterized by osteolytic bone resorption. Whether and how Wnt/ β -catenin signaling plays roles in regulating the invasion, metastasis and osteolytic process of ccRCC to bone remain unclear. This study investigated the expression of DKK1, LRP5 proteins in primary and metastatic lesions of RCC-BM. The therapeutic potential of Wnt/ β -catenin signaling target medication was also evaluated.

Materials And Methods

1. Patients and materials

In this retrospective analysis, ccRCC-BM patients with paired samples of primary and metastatic lesions were selected. Those patients were referred to department of urology and bone tumor center from Feb. 2016 to Nov. 2017. The same amount of ccRCC patients without any metastasis (ccRCC-only) during at least 2-year follow up were set as control. After the approval from institutional ethical committee, the study was conducted retrospectively on thirty-four formalin-fixed paraffin embedded tissue blocks of ccRCC primary and metastatic lesions. The blocks were obtained from the archival biobank, department of pathology, People's Hospital, Peking University. Slides of paraffin-embedded tissue were prepared for the further hematoxylin and eosin staining and/or immunohistochemical staining investigation.

The clinical features were retrieved from patients' medical records, including their age, gender, and tumor size. Hematoxylin and eosin staining of RCC samples was used to examine the tumor specific features including tumor stage (according to American Joint Committee on Cancer /AJCC criteria) [15], nuclear grade (according to International Society of Urological Pathology/ISUP grading system) [16]. MSKCC/Motzer score was selected for prognostic prediction in ccRCC-BM patients [17]. Patients with 0 points would be stratified to the favorable risk group. Patients with 1-2 points were set to the intermediate risk group. Patients with 3 or more points were set to the poor risk group.

2. Immunohistochemistry

Immunohistochemical staining was performed as the following method:

Tissues of 4 μm thickness were taken from paraffin-embedded blocks. The slides were then de-waxed and rehydrated through descending graded ethanol series down to distilled water. The rehydrated sections were treated with 3% hydrogen peroxide for 10 min to block the endogenous peroxidase. For epitope retrieval, sections were heated in citrate buffer (1:50 diluted, pH 8) using water bath for 25 min. Non-specific staining had been blocked by goat serum for 25 min. Sections were incubated with the primary antibodies overnight in a 4 degrees Celsius environment. The antibodies used were rabbit monoclonal anti-DKK1 antibody (Abcam, ab109416, UK, diluted at 1:100) and polyclonal anti-LRP5 antibody galectin-3 ((Abcam, ab38311, UK, diluted at 1:50). Secondary antibody and chromogenic reagent (3,3'-diaminobenzidine/DAB) kits were used according to the manufacturer's instructions (ZSGB-BIO Corporation, China). Counter staining was done with Mayer's hematoxylin and sections then are examined by light microscopy (DM2500, Leica, Germany).

3. Evaluation of DKK1 and LRP5 expression

Semi-quantitatively scoring according to staining intensity was performed. Two physicians judged the results blinded. The results were determined from the following two dimensions:

- 1) Reactivity: defined as membranous, cytoplasmic or nuclear stain magnitude, 0 points for no coloring, 1 point for light yellow, 2 points for yellow, 3 points for brown.
- 2) Staining range: defined as the proportion of positive cells in the selected staining area (such as renal tissue adjacent to renal tumor, primary renal tumor, bone metastasis).

For each slide and area, the staining result was calculated by multiplying scores of the above two dimensions. Staining results in the renal tissue adjacent to RCC, the primary RCC lesions (with BM or without BM), and the RCC-BM lesions were recorded separately.

4. Statistical analysis

The expression difference was analyzed by Chi squared test and univariate analysis of variance (ANOVA). Those analyses were conducted using SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA). A two-sided p value of less than 0.05 was set as the cutoff for statistical significance.

Results

Patient characteristics and tumor-specific characteristics are shown in Table 1. The baseline characteristics including average age and tumor size, pathological T stage, ISUP grade determined post-operatively in the RCC-BM group with were nearly same with that of RCC group.

Table 1
Patients' demographic and clinical features

	RCC-BM	RCC-only	P-value
N	11	12	
Age	55.45±7.93	54.08±13.06	0.766
Gender(male)	10(90.91%)	9(75.00%)	0.590
MSKCC Stratification			
Low risk	0		
Intermediate risk	9(81.82%)		
High risk	2(18.18%)		
T1+T2/T3+T4	5/6	4/8	0.680
ISUP Grade	7/4	9/3	0.667
Low (1+2)/High (3+4)			

The expression of DKK1 was seen mainly on the cell membrane, cytoplasm, and seldomly, on nucleus (Figure 1). Significant difference was found amid renal tissue adjacent to RCC, primary RCC and RCC-BM tissues ($p < 0.001$, figure 2). The expression of DKK1 in primary RCC lesions was significantly lower than that in renal tissue adjacent to RCC ($p < 0.001$) with no difference between ccRCC-BM group and ccRCC-only group. Notably, in bone metastasis, DKK1 expression was significantly higher than that in primary tumor ($p < 0.001$).

The LRP5 protein was mainly seen on cell membrane (Figure 3). For ccRCC-BM patients, the expression of this molecule in the primary tumor was significantly lower than that in adjacent renal tissue ($p < 0.01$). Tendency of decreasing expression was found between primary lesion of ccRCC-BM group and primary lesion of ccRCC-only group ($p = 0.073$, figure 4). In bone metastasis, the expression of LRP5 protein was not significantly different from that in adjacent renal tissue and RCC primary lesion.

Discussion

The bone homeostasis is maintained by the balance between osteoblasts and osteoclasts. Malignant bone metastasis, appearing in the form of osteoblastic reaction, osteolytic destruction or the mixed, is the results of bone metabolism disequilibrium. Bone metastasis characterized by osteolytic destruction illustrates the over-differentiation and functional enhancement of osteoclasts. Beyond the three well-known theories [18–20], which include 1) over-activation of nuclear factor κ B activated receptor/ligand (RANKL/RANK) signaling, 2) over-stimulation of monocyte by macrophage colony stimulating factor (M-CSF) to differentiated into osteoclast, and 3) the local pro-inflammatory environment enhancing the osteolysis process mediated by osteoclast, the Wnt signaling has recently been identified to affect bone

homeostasis [21]. Inhibition of this signaling is related to the development of fractures, osteoporosis, osteogenesis imperfecta and other diseases that manifest as osteopenia and bone destruction.

The Wnt/ β -catenin signaling involves in the development of a variety of malignancies [22, 23]. After binding to the receptors on cell membrane, Wnt protein could mediate the aggregation of β -catenin in the cytoplasm and further promote nuclear gene transcription [24]. In ccRCC, the activation of the Wnt/ β -catenin signaling has long been recognized to promotes tumorigenesis [25, 26]. The up-regulated expression of Wnt1, Wnt10 protein in RCC tissues positively correlated to greater tumor diameter, tumor progression and invasiveness [27, 28]. As an antagonist to Wnt signaling, the expression of DKK family molecules (including DKK1-4) was down regulated in RCC tissues [29–31].

In the current study of ccRCC, the expression of this Wnt/Beta-catenin signaling inhibitor, DKK1, was lower in primary renal tumor tissue than that in adjacent renal tissues. This correlated with the RCC carcinogenesis promoting by Wnt/ β -catenin signaling activation. The expression of DKK1 in the osteolytic bone metastasis lesion was up-regulated comparing to primary lesion, which suggests the association between Wnt/ β -catenin signaling inhibition and bone destruction in ccRCC-BM.

Further, in previous studies on lipid metabolism, oxidized low-density lipoprotein (ox-LDL) was found to induce the DKK1 expression and further promote the exocytosis of lipid components [32]. Our previous study also demonstrated that the serum LDL level in patients with RCC-BM was significantly higher than that of patients without any metastasis [33]. The up-regulated expression of DKK1 in bone metastasis lesions was in line with the increasing LDL level. Meanwhile, LRP5 is a component of Wnt receptor on cell membrane, as well as the target of DKK1 inhibition in Wnt signaling [34]. As a kind of LDL receptor, LRP5 receptor promoted the endocytosis of LDL and its expression was negatively correlated with the serum LDL level [35, 36]. Those propose the possible connections among the abnormal lipid metabolism, the invasion of ccRCC to bone and the Wnt/ β -catenin signaling inhibition by DKK1 through LRP5.

Down-regulation of LRP5 expression would reduce bone mass [37]. This down-regulation is caused by genetic changes such as gene polymorphisms and mutations [38]. In this study, the expression of LRP5 was significantly down-regulated primary renal lesion of patients with bone metastases, which suggested that RCC patients with low LRP5 expression might be more prone to bone metastasis. Since no differences of LRP5 expression were found between bone metastasis and primary RCC lesion, the promotion of bone destruction caused by DKK1 in RCC-BM might not be is not achieved by the inhibition of LRP5 receptor. Considering the metabolism of LDL is also regulated by other receptors such as LDL receptor (LDLR), further efforts to evaluate the regulation degree of LRP5 receptor in LDL metabolism are needed.

Inhibition of DKK1 provides new solution to treating osteolytic bone metastasis. is. A number of preclinical experiments and clinical trials on multiple myeloma, another malignancy characterized by osteolytic destruction, have shown that the anti-DKK1 antibody could suppress the osteoclast function, increase bone mass by neutralizing DKK1 [39–41]. In solid tumors, such as breast cancer, combination of zoledronic acid and aromatase inhibitor reduced serum DKK1 level in patients with bone metastasis [42,

43]. Although ccRCC-BM shares similarity to the above malignancies, considering the role of Wnt signaling in its carcinogenic process, further investigation is still needed to figure out the efficacy and adverse reactions of anti-DKK1 treatment in bone destroying prevention.

The number of cases limited the interpretation of results. But samples in this study were well paired specimens of renal tissue adjacent renal tissue, primary RCC and RCC-BM tissue, which is more helpful to discover roles of the same molecule in the whole course of disease. The mechanism behind the DKK1 and LRP5 expression is worth further probing. In vivo model of bone metastasis might need to be established.

Conclusion

A "rebound" of DKK1 expression was found in bone metastasis lesions of clear cell renal cell carcinoma. Along with the decreasing LRP5 expression in primary lesions of ccRCC-BM patients, this suggests that the canonical Wnt signaling (Wnt/ β -catenin signaling) is inhibited during the bone metastasis process in ccRCC. The overexpression of DKK1 and the down-regulation of LRP5 receptor are involved. Those molecules might become the targets in the new generation of therapy.

Abbreviations

BM	bone metastasis
RCC	renal cell carcinoma
ccRCC	clear cell renal cell carcinoma
TKIs	tyrosine kinase inhibitors
DKK1	Dickkopf 1
LRP5	low density lipoprotein related protein 5
LDL-C	low density lipoprotein cholesterol
LDLR	low density lipoprotein receptor
AJCC	American Joint Committee on Cancer
ISUP	International Society of Urological Pathology
MSKCC	Memorial Sloan-Kettering Cancer Center
RANK	nuclear factor κ B activated receptor
M-CSF	macrophage colony stimulating factor
ANOVA	univariate analysis of variance

Declarations

- **Availability of data and materials**

The data and materials used and analyzed in this current study are stored in the data system of Peking University People's Hospital and are available from the corresponding author on reasonable request.

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- **Ethics declarations**

Ethics approval and consent to participate

This study was conducted with the approval of Peking University People's Hospital review board. The study-specific informed consent is waived according to the approval document.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interest.

- **Authors' contributions**

Z Huang: Conceptualization, Methodology, Investigation, Writing; Y Du: Conceptualization, Validation; H Yin: Methodology; G Wang: Validation, Resources; Tao Xu: Conceptualization, Supervision, Review & Editing.

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Figures

Figure 1

The immunohistochemical staining results of DKK1 in renal tissue adjacent to RCC, primary RCC and RCC-BM tissues

Figure 2

The relative expression of DKK1 in renal tissue adjacent to RCC, primary RCC and RCC-BM tissues

Figure 3

The immunohistochemical staining results of LRP5 in renal tissue adjacent to RCC, primary RCC and RCC-BM tissues

Figure 4

The relative expression of LRP5 in renal tissue adjacent to RCC, primary RCC and RCC-BM tissues