# Analysis of the EPAS-1 Expression Level and Pathological Characteristics of Renal Cell Carcinoma

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## ABSTRACT

This study aims to analyse the expression level of the endothelial PAS domain protein 1 (*EPAS-1*) gene in renal cell carcinoma (RCC), adjacent tissue and metastatic lymph node tissue. For this study, 110 cases of RCC tissue and corresponding adjacent tissue and 40 cases of metastatic lymph node tissue were selected. Western blot and immunohistochemical methods were used to detect the expression of EPAS-1 in the three groups. The expression of EPAS-1 and the clinicopathology of RCC were further analysed. To examine the relationship between these characteristics, a receiver operating characteristic curve (ROC) was used to evaluate the diagnostic value of *EPAS-1* expression for lymph node metastasis in RCC. Compared with the adjacent tissue, the expression of EPAS-1 in RCC was significantly increased ($P < 0.05$, $\chi2 = 4.311$). The correlation between the expression of EPAS-1 and primary tumour staging, lymph node metastasis, distant metastasis and the International Society of Urological Pathology (ISUP) grade of clear cell RCC patients were analysed. The classification using ISUP was significantly correlated with the expression of EPAS-1 ($P < 0.05$). The ROC curve showed that the high expression of EPAS-1 has a certain diagnostic value for lymph node metastasis in RCC ($P < 0.01$). To conclude The high expression of EPAS-1 is closely related to the adverse clinicopathological characteristics of RCC and can be used as a potential therapeutic target for this disease. The detection of the EPAS-1 gene has an auxiliary diagnostic value for lymph node metastasis in RCC.

## INTRODUCTION

Renal cell carcinoma (RCC) is the most common malignant tumour in the urinary system (Hota *et al*., 2020; Morton *et al*., 2020) and is harmful to human health. After years of research, the pathogenesis of RCC is not completely clear (McInnis and Pukall, 2020; Ritter *et al*., 2020). Therefore, investigating its pathogenesis is important for diagnosing and treating this disease. A hypoxic microenvironment is common in solid tumours (Fragkoulis *et al*., 2020). Endothelial PAS domain protein 1 (EPAS-1), also known as hypoxia-inducible factor (HIF) 2α, is widespread in the mammalian body, plays a central role in hypoxic reactions and participates in the pathological processes of many diseases. As a member of the HIF family, HIF-1α transcription primarily regulates metabolic reprogramming, whereas EPAS-1 exerts a larger role in regulating angiogenic extracellular signalling guidance cues and extracellular matrix remodelling factors. Furthermore, EPAS-1 almost exclusively regulates a large and diverse subset of transcription factors and coregulators that contribute to its diverse roles in hypoxia. As a type of transcription factor, EPAS-1 hypoxia is related to tumour growth, metastasis and angiogenesis. Meanwhile, hypoxia and tumour angiogenesis play a significant role in tumour growth, invasion and metastasis. Tumours form new blood vessels by adapting to the ischemic and hypoxic environment (Christian *et al*., 2019; Das *et al*., 2020; Heaton *et al*., 2019). Furthermore, EPAS-1 can promote the production of angiogenic factors (Rosenblum *et al*., 2019).

In this study, Western blot and immunohistochemical methods are used to detect the expression of EPAS-1 in RCC and adjacent and metastatic lymph nodes to

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investigate the correlation between the expression of EPAS-1 and the pathological features of RCC. A receiver operating characteristic (ROC) curve is used to determine the value of EPAS-1 for RCC.

## MATERIALS AND METHODS

### General information

In this study, 110 cases of confirmed RCC and corresponding adjacent tissue and 40 cases of metastatic lymphoid tissue were collected at the Affiliated Hospital of Hebei Engineering University from February 2016 to February 2020. This experiment was confirmed by pathology, and no tumour cells were found at the surgical excision position incisal margin. All specimens were fixed with 10% neutral formalin, embedded in paraffin and sectioned continuously (approximately 4 µm thick). Of the 110 patients with RCC, there were 75 male and 35 female patients, aged 40 to 80 years, with a median age of 65 years. None of the patients had received corresponding treatment measures before surgery, such as radiotherapy, chemotherapy or biotherapy.

### Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) patients had been diagnosed with RCC; (2) clinicopathological data of patients had been well preserved; (3) participants had been newly diagnosed; (4) patients had not undergone chemotherapy and/or radiotherapy before participating in the study; and (5) patients had provided signed informed consent.

The exclusion criteria were as follows: (1) patients with other types of malignant tumours; (2) patients with an autoimmune disease; (3) patients with a dysfunction of vital organs or an infectious disease; and (4) patients without pathological data.

### Methods

To carry out the Western blot analysis, total protein was extracted from patient tissues. The total protein amount was determined by bicinchoninic acid assay (Thermo), and an appropriate amount of denatured protein with Lammeli loading dye was loaded onto 10% polyacrylamide gel for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The polyvinylidene fluoride membrane was used for transfer, and a tris-buffered saline-Tween buffer containing 5% bovine serum albumin with 0.02% sodium azide was used in membrane blocking and antibody incubations. The primary antibody used was rabbit anti-human EPAS-1 monoclonal antibody (1:1000, Cell Signaling Technology, 71565), and mouse anti-human glyceraldehyde 3-phosphate dehydrogenase

monoclonal antibody (1:1000, Cell Signaling Technology, 5174) was used as a loading control. The secondary antibodies (Cell Signaling Technology, 7074) were used in a 1:5000 dilution.

Immunohistochemical staining was performed using the EnVision two-step method. The staining steps were as follows: (1) the sections were dewaxed for hydration, (2) 3% hydrogen peroxide was used to block endogenous peroxidase activity and (3) a high-pressure pot method was used for antigen retrieval. Normal serum was used for incubation, and the anti-human antibody EPAS-1 (1:200) was added and kept overnight at 4°C. Then, the second antibody of EnVision and 3,3′-diaminobenzidine colouration was added, followed by hematoxylin re-dyeing, dehydration, transparency and sealing. Phosphate-buffered saline and homologous mouse immunoglobulin G were used as negative controls.

### Evaluation method

At least five visual fields were randomly observed in a 200× magnification visual field, and positive results were indicated by yellow or brown granules in the nucleus or cytoplasm. A double-blind experiment was performed in this study, and a semi-quantitative integral method was applied, using the product of the positive cell rate and staining intensity score as the total score. A score of less than three was determined to be negative, and more than three was positive. According to dyeing intensity, staining intensity was divided as follows: Zero points for no staining, one point for weak-intensity staining, two points for medium-intensity staining and three points for high-intensity staining. According to the percentage score of stained cells, zero points was 0%, one was 1%–25%, two was 26%–50%, three was 51%–75% and four was 76%–100%. In this study, statistics were only applied with negative tests (zero points for the staining intensity score and zero points for the percentage) and the others were determined to be positive. Univariate analysis was performed for the following pathological factors: multifocality tumour size, primary tumour stage (pT), lymph node stage, distant metastases, pathological stage (tumour node metastasis [TNM], 1997 and Robson's classification) and nuclear grading of tumour (Fuhrman's classification). Pathological stages of RCC were classified according to the American Joint Committee on Cancer (Williamson *et al.*, 2019).

### Statistical analysis

In this study, SPSS 25.0 software was used for statistical analysis. The expression of EPAS-1 in RCC, adjacent tissue and lymph node tissue was compared using the $x^2$ test, and the correlation between the expression

of EPAS-1 and the clinicopathology was analysed. A ROC curve was used to evaluate the diagnostic value of the expression of EPAS-1 in RCC, and $P < 0.05$ was statistically significant.

## RESULTS

*The expression of EPAS-1 in renal cell carcinoma, adjacent tissue, and metastatic lymph nodes*

The results of this study demonstrated that the EPAS-1 staining increased gradually in adjacent tissue, RCC tissue and metastatic lymphoid tissue (Figs. 1A–C). The Western blot result showed the same conclusion (Fig. 1D). The EPAS-1 expression rate in RCC was 64.5%, which was significantly higher than that of the adjacent tissue (14.5%), and the difference was statistically significant ($\chi2 = 4.311$, $P < 0.05$). The EPAS-1 expression level in metastatic lymph nodes was the highest of the three groups, and the positive rate was 80% when compared with RCC and adjacent tissue. The difference was statistically significant ($\chi2 = 4.268$, $P < 0.05$) (Table I).
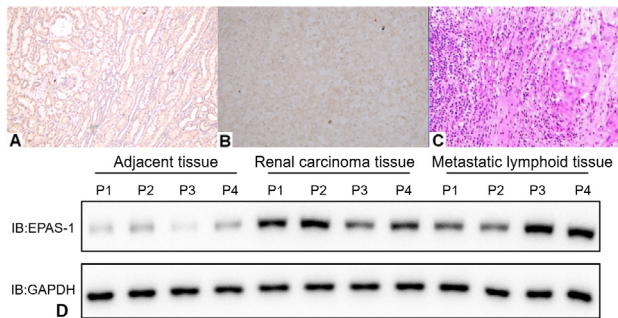


Fig. 1. Expression of EPAS-1 in adjacent tissues (A), in renal carcinoma tissues (B), in metastatic lymphoid tissues (C) and Western blot of expression of EPAS-1 in adjacent tissues, renal carcinoma tissues and metastatic lymphoid tissues (D).

*EPAS-1 expression in renal cell carcinoma and the relationship between EPAS-1 and clinicopathological features*

The results of this study showed that the expression of EPAS-1 was not related to age, gender or the RCC pT stage, but it did correlate with the International Society of Urological Pathology (ISUP) grade, lymph node metastasis, distant metastasis and TNM stage ($P < 0.05$) (Table II).

*The value of endothelial PAS domain protein 1 for diagnosis of renal cell carcinoma*

In this study, a ROC curve was used to evaluate the role of EPAS-1 in diagnosing RCC. The results demonstrated that the EPAS-1 had a certain value in the diagnosis of RCC (95% confidence interval: 0.572–0.708, $P < 0.001$), with an area under the curve (AUC) of 0.640 (Fig. 2).
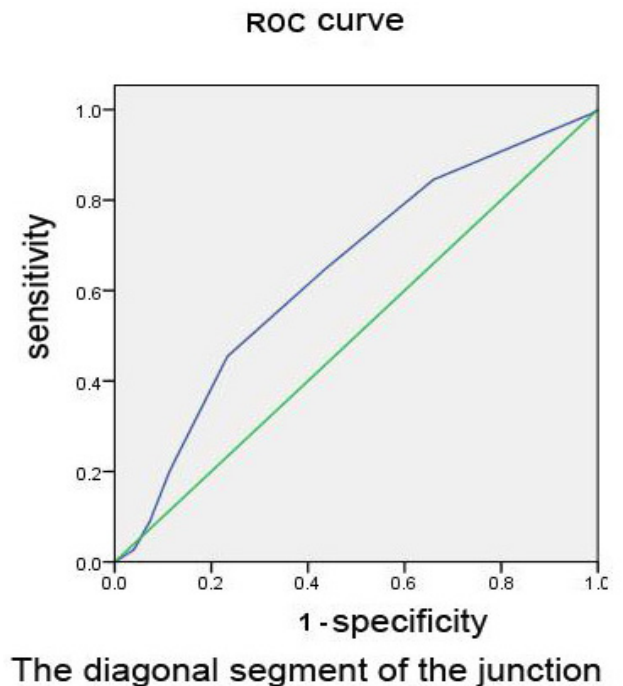


Fig. 2. The diagonal segment of the junction.

## Table I. Expression of EPAS-1 in renal cell carcinoma, adjacent tissues and metastatic lymph nodes.

| Tissue | N | EPAS1 | | $X^2$ | p |
|---|---|---|---|---|---|
| | | Negative | Positive | | |
| Adjacent tissues | 110 | 94(85.5%) | 16(14.5) | 4.311[a] | 0.038[a] |
| Renal cell carcinoma tissues | 110 | 39(35.5%) | 71(64.5%) | 4.268[b] | 0.039[b] |
| Metastatic lymphoid tissues | 40 | 8(20%) | 32(80%) | 30971[c] | 0.046[c] |

[a] adjacent tissues vs. renal cell carcinoma tissues; [b] renal cell carcinoma tissues vs. metastatic lymphoid tissues; [c] adjacent tissues vs. metastatic lymphoid tissues.

**Table II. Expression of EPAS-1 in renal cell carcinoma and the relationship between EPAS-1 and clinicopathological features.**

| Groups | N | EPAS-1 | | X² | P |
|---|---|---|---|---|---|
| | | Negative | Positive | | |
| **Age** (Years) | | | | 0.083 | >0. 05 |
| ≤60 | 39 | 12(30.77%) | 27(69.23%) | | |
| > 60 | 71 | 20 (28.17%) | 51(71.83) | | |
| **Sex** | | | | 0.031 | >0. 05 |
| Male | 75 | 27(36.0%) | 48(64.0%) | | |
| Female | 35 | 12(34.29%) | 23(65.71%) | | |
| **PT stage** | | | | 18.558 | P<0.01 |
| T1 | 57 | 30(52.63%) | 27(47.37%) | | |
| T2 | 26 | 7 (26.92%) | 19(73.08%) | | |
| T3 | 17 | 2(11.76%) | 15(88.24%) | | |
| T4 | 10 | 2 (20.0%) | 8(80.0%) | | |
| **Lymph node metastases** | | | | 4.610 | P<0.05 |
| Yes | 40 | 9(22.5%) | 31(77.5%) | | |
| No | 70 | 30(42.86%) | 40(57.14%) | | |
| **Distant metastasis** | | | | 4.739 | P<0.05 |
| Yes | 8 | 2 (25.0%) | 6(75.0%) | | |
| No | 102 | 39(38.23%) | 63(6176%) | | |
| **ISUP stage** | | | | 11.811 | P<0.01 |
| I | 57 | 28(49.42%) | 29(50.88%) | | |
| II | 15 | 5(33.33%) | 10(66.67%) | | |
| III | 24 | 4(16.67%) | 20(8.33%) | | |
| IV | 14 | 2 (14.29%) | 12(85.71%) | | |

## DISCUSSION

Solid tumours exist in hypoxic microenvironments. EPAS-1 is a transcription factor that exists widely in the human body under hypoxic conditions and is related to tumour growth, metastasis and angiogenesis (Yao, 2019). It has been found that the positive expression of EPAS-1 was related to the clinical stage and size of the tumour. The later the clinical stage and the larger the tumour volume, the higher the positive expression of EPAS-1 (Wang *et al*., 2019). Due to the rapid proliferation of tumour cells, vascular abnormalities and anaemia, the formation of a hypoxic environment in solid tumours is a common feature (Ullrich *et al*., 2019). Studies have shown that intracellular hypoxia is closely related to the occurrence and development of solid tumours (Bechmann *et al*., 2019; Cruzeiro *et al*., 2018). The unique hypoxic microenvironment in solid tumours is not only related to tumour metabolism, angiogenesis and metastasis but also tumour cell resistance to radiotherapy and chemotherapy (Wang *et al*., 2017). It was reported that endogenous vascular endothelial growth factor could be up-regulated transcriptionally by EPAS-1, and EPAS-1 may be involved in the angiogenesis of RCC (Xia *et al*., 2001). EPAS-1 appears to promote cancer development and progression in neuroblastoma and renal carcinoma, and it is inversely associated with high tumour grades (Baba *et al*., 2010). A bioinformatics analysis of the microarray data screening of differentially expressed genes indicated that EPAS-1 was differentially expressed in RCC (Higgins *et al*., 2003), but there was no report on the relationship between the expression of EPAS-1 and the pathological features of renal clear cell carcinoma and its diagnostic value for lymph node metastasis.

This study used an immunohistochemical method to detect the expression of EPAS-1 in RCC and adjacent and metastatic lymph nodes. The study found that the expression of EPAS-1 in RCC was significantly higher than that of adjacent tissues, and the expression level of EPAS-1 in RCC metastatic lymph nodes was significantly higher than that of RCC. This validates the results of the bioinformatics analysis of the microarray data screening of differentially expressed genes. At the same time, it also shows the genetic changes of EPAS-1 after the malignant transformation of renal cells. Further study showed that the differential expression of EPAS-1 was significantly correlated with the ISUP grade, pT stage, lymph node metastasis and distant metastasis. These results suggest that EPAS-1 plays a key role in the invasion and metastasis of RCC. In addition, a ROC curve was used to evaluate the diagnostic value of the EPAS-1 expression in RCC, and its AUC was 0.640, indicating that EPAS-1 has a certain diagnostic value in RCC.

However, the invasion and metastasis mechanism of RCC is complicated; it involves the interaction between tumour cells, host cells and the internal environment, which needs to be further studied using cell and animal models. In addition, a limitation of this study is that it only investigated the relationship between EPAS-1 in RCC and its clinicopathological features and the value of EPAS-1 expression in RCC diagnosis; however, the study did not investigate the possible signal transduction pathway, which still needs further research at the cellular level. Moreover, the number of patient samples in this study was small, and the diagnostic value of EPAS-1 has not been used in clinical practice. More cases will be collected to verify this study's conclusion and the role of EPAS-1 in diagnosing RCC.

## ACKNOWLEDGEMENTS

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