10.1053.paor.2001.0295 available online at http://www.idealibrary.com on IDEAL

ARTICLE

Specific von Hippel-Lindau Protein Expression of Clear Cell Renal Cell Carcinoma with "Immunogenic" Features*

Tamás MAGYARLAKI,[1] István BUZOGÁNY,[2] Judit NAGY[3]

[1]Department of Clinical Biochemistry, [2]Department of Urology and [3]2nd Department of Medicine, University Medical School of Pécs, Pécs, Hungary

Human clear cell renal cell carcinoma (CCRCC) is characterized by specific von Hippel-Lindau (VHL) gene alterations and "immunogenic" features. In the present study, the immunohistochemical expression of the von Hippel-Lindau gene protein (pVHL) was compared with the presence of major histocompatibility complex (MHC I-II), tumor infiltrating lymphocytes (TIL) and tumoral immune complexes (TIC) in CCRCC. Native tumor tissues of 132 RCC patients (95 with the common clear cell subtype), diagnosed according to the Heidelberg classification, were obtained for immunohistochemistry. Tumor stainings with pVHL, MHC I-II and tumor infiltrating lymphocytes (T and B lymphocytes, monocytes) were detected by immunoperoxidase methods using monoclonal antibodies. Tumoral immune complexes (IgG, IgA, IgM and C1q, C3 complement proteins) were visualized by fluorescent polyclonal antibodies. Immune stainings were semiquantitatively evaluated. Specificity and sensitivity of these markers in relation to the common histological subtype of RCC (CCRCC) were calculated. CCRCC was characterized by specific pVHL expression. At the same time, CCRCC was associated with constitutional MHC I-II expression and highly specific degree of TIL and TIC. It is concluded that specific pVHL expression of CCRCC is frequently associated with "immunogenic" features. Immunohistochemical analysis aims the initial tumor staging of RCC patients to achieve better patient selection for immunotherapy. However, the association of pVHL expression with the "immunogenic" CCRCC is statistically relevant, the mechanism and its clinical relevance in immunotherapy still remains to be tested. (Pathology Oncology Research Vol 7, No 1, 42–45, 2001)

Keywords: clear cell renal cell carcinoma, von Hippel-Lindau protein, tumor infiltrating lymphocytes, immune complexes

Introduction

Renal cell carcinoma (RCC) is a promising target for immunotherapy. The response rate should be enhanced by selecting patients expressing relevant tumor antigens with active antigen presentation through major histocompatibility complex (MHC I-II) and characterized by effective antitumor mechanisms (tumor infiltrating lymphocytes [TIL] and immune complexes [TIC]).[2,13,16] MHC-restricted T lymphocytes with specific cytotoxic activity for autologous tumor cells persist in some RCC patients for years after removal of the primary tumor even in the absence of immunotherapy.[8] Immunohistochemical studies proved a significant TIL infiltration of RCC by preoperative interferon-gamma (IFN-gamma) administration.[9,15] The phenotype of TIL isolated from RCC patients is heterogenous (predominant MHC class I-restricted CD8+ T lymphocytes; few gamma/delta T cells, monocytes and NK cells).[11] Activation markers (CD69, CD25, HLA-DR) are expressed on TIL, but autologous tumor lytic activity is impaired in most of the RCC patients.[6,7] The contribution of cytokines and co-

Received: Sept 11, 2000; *revised:* Dec 20, 2000; *accepted:* Jan 15, 2001
Correspondence: Tamás MAGYARLAKI, Department of Clinical Biochemistry, University Med.School of Pécs, Ifjúság útja 13, 7624 Pécs, Hungary; Tel: +36-72-536-120 Fax: +36-72-536-120 Email: mlaki@clinics.pote.hu
*This work was supported by the grants of the Hungarian Academy of Sciences (BOLYAI-BO/00049/99 and OTKA-T0220986)

Abbreviations:
CCRCC: clear cell renal cell carcinoma; RCC: renal cell carcinoma; VHL: von Hippel Lindau gene; pVHL: protein; TIL: tumor infiltrating lymphocytes; TIC: tumoral immune complexes; MHC: major histocompatibility complex; MHC-I and II: Class I and II.

 1219-4956/01/010042+04 $ 12.00/0

stimulatory molecules in TIL-tumor cell interaction is under evaluation.[7] The clinical relevance of intratumoral lymphocytic invasion is still controversial in interleukin-2 (IL-2) and TIL-based therapy.[1,2,16]

Routine histology does not improve on prediction of antitumor response in RCC. Some studies, however, found MHC-I expression,[4,12] TIL[6,14] and TIC[13,14] exclusively in the clear cell subtype of RCC. This "immunogenic" subtype of RCC (the typical clear cell renal tumor of von Hippel-Lindau [VHL] syndrome and 75% of sporadic renal neoplasms) was separated from others on the basis of specific genetic alterations on chromosome 3p.[3,10] Mutations of a single allele of the VHL gene are associated with deletion of the remaining functioning VHL allele ("loss of heterozygosity"). Recent development of monoclonal antibodies against VHL protein (pVHL) enabled the detection of tumoral pVHL expression by immunohistochemistry.[5] Since pVHL is known as an antigen of RCC-associated paraneoplastic immune complex nephropathies,[14] mutant pVHL can be a target antigen of antitumor immunity and specific immunotherapy.

In the present study pVHL expression and immunohistochemical markers accepted as tags of immunotherapy (MHC I or II, TIL and tumor immune complexes) were compared in 132 RCC patients.

Materials and Methods

Patients

Native tumor tissues with adjacent renal parenchyma were obtained from 132 resected kidneys of RCC patients. The histology according to the Heidelberg classification[10] revealed a subtype distribution (common clear cell, 72%; sarcomatous, 5%; mixed, 6%; chromophobe, 3%; papillary, 7%; renal oncytoma, 7%); pathological grade (I-II, 73%; III-IV, 17%); stage (Std I-II, 68%; III, 24%; IV, 8%) and metastatic cases (22%) comparable with those in the literature.[16]

Immunohistochemistry

Stainings was performed on acetone-fixed cryostat sections. For demonstration of TIC, fluorescent isothiocyanate (FITC)-labeled polyclonal rabbit antihuman IgG, IgA, IgM and complement (C1q, C3) antibodies (DAKO, diluted 1:10) were used.[14] An immunoperoxidase method using a secondary biopolymer reagent (Envision kit, DAKO) detected pVHL (Pharmingen monoclonal IG32, 0.5 mg/ml, recognizing amino acids 1-213 of human pVHL).[5] The ABC-immunoperoxidase method detected TIL (UCHL-1 for "memory T lymphocytes", Mac387 for monocytes, CD45RB for B lymphocytes), the β-chain of MHC-I (β2-microglobulin) and HLA-DR -chain of the MHC-II (DAKO monoclonals, dilution 1:50).[13]

Evaluation of the immunohistochemistry and statistical methods

Immunohistochemical staining was graded microscopically by two independent observers as described before in detail.[13] Briefly, tumor tissues of RCC cases with mild, focal or negative staining, were estimated as negative, in contrary to cases with moderate to strong diffuse positivity categorized as positive. Tumor infiltrating lymphocytes were counted in 20 microscopic fields of an 0.05 mm^2 eyepiece graticule at x200 magnification. Cases with marginal immune cell infiltration or intratumoral TIL below 100 cells/mm^2 were estimated as negative. In contrast, RCC cases with TIL present exclusively in the tumor area and exceeding 100 cells/mm^2 were positive. Specificity and sensitivity of these immunohistochemical markers in relation to the common clear cell histological subtype of RCC were calculated as follows:

Specificity = true negative cases / true negative + false positive cases x 100,

Sensitivity = true positive cases / true positive + false negative cases x 100.

As an example, by calculating the specificity and sensitivity of pVHL staining in relation to CCRCC histological subtype, all CCRCC cases stained positive with pVHL were "true positive", while CCRCCs with negative pVHL staining were estimated as "false negative". Renal cell carcinomas with non-CCRCC morphology, which exhibited negative pVHL staining, were estimated as "true negative", while the remaining cases with pVHL positivity, as "false positive". The specificity and sensitivity of the other markers were calculated similarly.

Results

Von Hippel-Lindau protein (pVLH) was exclusively present in RCC with clear cell morphology (CCRCC) (*Table 1*, specificity). *Figure 1* presents the typical clear

Table 1. **Statistical analysis of immunohistochemical (IHC) markers in relation to the common clear cell subtype of renal cell carcinoma (CCRCC).**

IHC staining	*CCRCCs / total RCCs tested*	*specificity (%) to CCRCC*	*sensitivity (%) to CCRCC*
pVHL	68 / 95	100	91
MHC-I	55 / 76	25	91
MHC-II	63 / 86	32	98
TIL	95 / 132	96	45
TIC	87 / 121	89	55

pVHL = von Hippel-Lindau protein, MHC-I and II = major histocompatibility complex, types-I and II, TIL = tumor infiltrating lymphocytes, TIC = tumoral immune complexes.

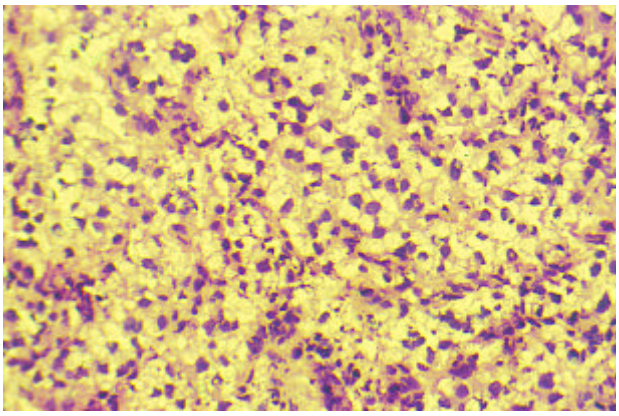

Figure 1. Clear cell renal cell carcinoma (CCRCC). A characteristic acinar tumor growth of the typical tumor cells with "clear cell" morphology. HE staining. x250.

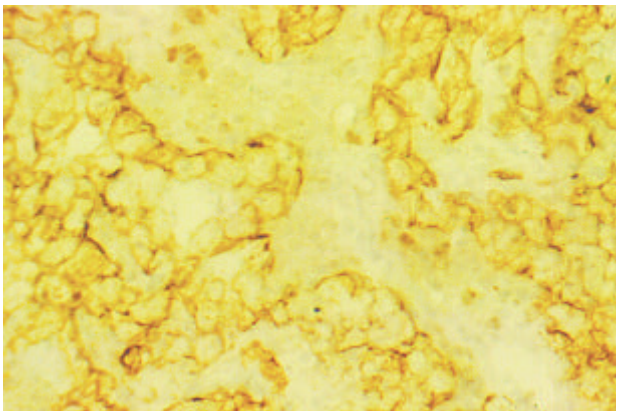

Figure 2. Von Hippel-Lindau protein (pVHL) expression of a CCRCC. A fine linear superficial staining of the tumor cells arranged in the acini is well seen. Immunoperoxidase staining with a pVHL monoclonal antibody. x250.

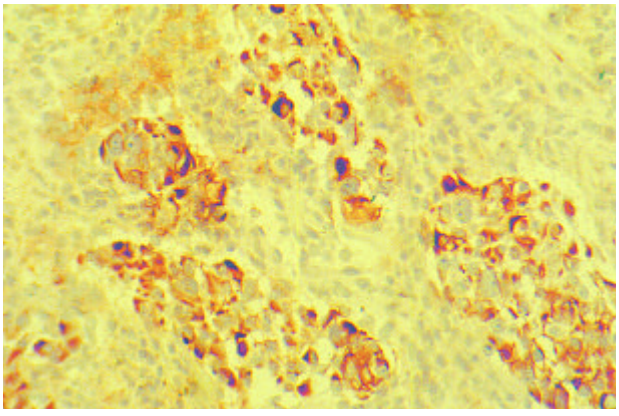

Figure 3. Von Hippel-Lindau protein (pVHL) expression of a grade III CCRCC. A strong cytoplasmic positivity of the tumor cells in some foci of a diffuse sarcomatous tumor parenchyma is visible. Immunoperoxidase staining with a pVHL monoclonal antibody. x250.

cell histological pattern of CCRCC morphology. Most of CCRCCs were stained by pVHL on the tumor cell surfaces diffusely by immunoperoxidase reaction (*Figure 2*). Exceptionally, a strong focal cytoplasmic pVHL positivity of some localized areas (*Figure 3*) was observed in 3 grade III CCRCC cases. Only 6 out of 68 CCRCCs (4 of them with poorly differentiated sarcomatous parts) were negative for pVHL (*Table 1*, sensitivity, "false negatives"). All oncocytomas (n=9), papillary and chromophobe (n=12) tumors were pVHL negative (specificity, "true negatives"). The clear cell component – but not of the oncocytomatous, papillary or chromophobe part) – of mixed type RCC (n=3;2;1, respectively) was also positive for pVHL (data not shown). Tumor MHC-I and II expression was simultaneously present in most of the CCRCCs (sensitivity). The specificity was, however, low (16 out of 21 and 15 out of 23 renal cell carcinomas with non-CCRCC morphology were "false positive" for MHC I and MHC II, respectively). TIL and/or TIC were highly specific for CCRCC, but present in only half the cases (specificity and sensitivity). RCC subtypes other than that of the "common" CCRCC type, were rarely "false positive" for TIC (in 3 oncocytomas) or TIL (in 1 papillary RCC). No correlation of "immunogenic" RCCs with grade, stage and metastasis was observed.

Discussion

Related to the specific mutations of the VHL gene,[3,10,16] the protein poduct (pVHL) is readily detectable by immunohistochemistry in the clear cell type of renal cell carcinoma (CCRCC). In contrast with the cytoplasmic pVHL positivity of renal proximal tubules,[5] we have found an almost consistent (except 3 cases) cell surface staining of pVHL in CCRCC. An "immunogenic" potential of the "superficialized" pVHL to induce RCC-associated paraneoplastic immune complex nephropathy has recently been published.[14] Mutant pVHL may either be a target antigen of antitumor immunity, or as a specific gene product of the CCRCC subtype modulate the expression of other relevant tumor antigens by the failure of its transcriptional regulatory function.[3,5,16] Further studies are needed to prove or exclude the causal relationship between pVHL expression and antitumor immunity.

"Immunogenic" features are restricted to CCRCC in many studies.[3,4,6,12-14] In our study, the well known constitutional tumoral MHC class I or II expression[2,4] was accompanied with TIL and TIC presence in CCRCC cases. While no patients with preoperative cytokine-therapy[9,15] were included in the study, these results reflect the status of the tumor immune recognition in RCC patients at the time of tumor removal. Impaired TIL function[6,7] is probable in our cases (massive TIL infiltration without associated histological signs of tumor necrosis). However,

significant correlation between the advanced tumor stage and genetic VHL alterations has recently been reported,[3] in our study the pVHL expression, the degree of TIL or TIC was independent of tumor grade, stage and the presence of metastasis. The prognostic relevance of these findings to immunotherapy[1,2,16] remains to be tested.

Conclusions

Renal cell carcinoma of the common clear cell type (CCRCC) is characterized by mutant von Hippel-Lindau protein (pVHL) expression. Constitutional MHC class I and II expression with highly specific degree of TIL and tumoral immune complexes render the CCRCC subtype to be "immunogenic". Mutant pVHL is either a potential target antigen of antitumor immunity or it may modulate the expression of other relevant tumor antigens. Immunohistochemical analysis aims the initial tumor staging of RCC patients to achieve better patient selection for immunotherapy.

Acknowledgement

We wish to thank the aim of Prof. Gyula Kovács in the histological reclassification of the RCC cases and the technical assistance of Ildikó Udvarácz, Tünde Wéber and Mária Kereszturi in the performance of histological and immunohistochemical techniques.

References

1. *Belldegrun A, Tao CL, Kaboo R, et al:* Natural immune reactivity-associated therapeutic response in patients with metastatic renal cell carcinoma receiving tumor-infiltrating lymphocytes and interleukin-2-based therapy. J Immunother Emphasis Tumor Immunol 19:149-161, 1996.
2. *van Bezooijen RL, Goey H, Stoter G, et al:* Prognostic markers for survival in patients with metastatic renal cell carcinoma treated with interleukin-2. Cancer Immunol Immunother 43:293-298, 1996.
3. *Brauch H, Weirich G, Brieger J, et al:* VHL alterations in human clear cell renal cell carcinoma: association with advanced tumor stage and a novel hot spot mutation. Cancer Res 60:1942-1948, 2000.
4. *Buszello H, Ackermann R:* Immunohistochemical studies on the expression of HLA Class I antigen in renal cell carcinoma: comparison of primary and metastatic tumor tissue. Eur Urol 25:158-163, 1994.
5. *Corless CL, Kibel AS, Iliopoulos O, et al:* Immunostaining of the von Hippel-Lindau gene product in normal and neoplastic human tissues. Hum Pathol 28:459-463, 1996.
6. *Van den Hove LE, van Gool S.W, van Poppel H, et al:* Identification of an enriched CD4+ CD8alpha++ CD8beta+ T-cell subset among tumor-infiltrating lymphocytes in human renal cell carcinoma. Int J Cancer 71:178-182, 1997.
7. *Van den Hove LE, van Gool SW, van Poppel H, et al:* Phenotype, cytokine production and cytolytic capacity of fresh (uncultured) tumor-infiltrating T lymphocytes in human renal cell carcinoma. Clin Exp Immunol 109:501-509, 1997.
8. *Jantzer P, Schendel DJ:* Human renal cell carcinoma antigen-specific CTLs: antigen-driven selection and long-term persistence in vivo. Cancer Res 58:3078-3086, 1998.
9. *Kawata N, Akimoto Y, Hirano, et al:* Immunological effect of recombinant interferon-gamma on tumor infiltrating lymphocytes of renal cell carcinoma – relationshship with clinical stage (Japanese). Hinyokika-Kiyo 42:1-4, 1996.
10. *Kovacs Gy, Akhtar M, Beckwith BJ, et al:* The Heidelberg classification of renal cell tumors. J Pathol 183:131-133, 1997.
11. *Kowalczyk D, Skorupski W, Kwias Z, et al:* Flow cytometric analysis of tumor-infiltrating lymphocytes in patients with renal cell carcinoma. Br J Urol 80:543-547, 1997.
12. *Licht MR, Novick AC, Tubbs RR, et al:* Renal oncocytoma: clinical and biological correlates. J Urol 150:1380-1383, 1993.
13. *Magyarlaki T, Mosolits S, Baranyay F, et al:* Immunohistochemistry of complement responses on human renal cell carcinoma biopsies. Tumori 82:473-480, 1996.
14. *Magyarlaki T, Kiss B, Buzogány I, et al:* Renal cell carcinoma and paraneoplastic IgA nephropathy. Nephron 82:127-130, 1999.
15. *Toliou T, Stravoravdi P, Polyzonis M, et al:* Natural killer cell activation after interferon administration in patients with metastatic renal cell carcinoma: an ultrastructural and immunohistochemical study. Eur Urol 29:252-256, 1996.
16. *Vogelzang NJ, Stadler WM:* Kidney cancer. Lancet 352:1691-1696, 1998.