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Impaired Vitamin D Signaling Is Associated With Frequent Development of Renal Cell Tumor in End-stage Kidney Disease

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Abstract. *Background/Aim: End-stage kidney disease is characterized by chronic inflammation and frequent development of cancer. The level of circulating vitamin D is generally low in patients with end-stage renal disease (ESRD). Experimental studies have implicated the role of dysfunctional vitamin D metabolism in tumorigenesis. Patients and Methods: We analyzed the expression of vitamin D receptor (VDR), cytochrome P450 family 27 subfamily B member 1 (CYP27B1) and cytochrome P450 family 24 subfamily A member 1 (CYP24A1), the key genes involved in vitamin D signaling, in kidneys from patients with ESRD, tissue microarrays containing ESRD-associated renal cell tumors, as well as in their precursor lesions by immunohistochemistry. Results: Kidneys from patients with ESRD showed strong structural rearrangement with only few tubules and epithelial cell groups embedded in fibrotic-inflammatory stroma. Only an estimated 1-3% of the epithelial cells showed positive staining with antibodies to VDR, CYP27B1 and CYP24A1, which contrasted with the 100%, 40-50% and 40-50% of positively stained cells, respectively, found in normal kidneys. Down-regulation of the vitamin D signaling proteins was found in patients with renal cancer, with the exception of tumors and their precursors occurring exclusively in ESRD. Conclusion: The significantly reduced activity of CYP27B1 in kidney from patients with ESRD explains the low level of circulating vitamin D. We suggest that the lack of anti-tumorigenic effect of vitamin D is a crucial factor in the frequent development of unique types of renal cell cancer in in patients with ESRD.*

The number of patients with diabetes mellitus and hypertension leading to chronic renal disease is rapidly growing worldwide (1). An increasing number of patients with type 2 diabetes receive renal replacement therapy in European Countries (2). This therapy prolongs the patient's life but, in several cases, leads to end-stage renal disease (ESRD), with major structural rearrangement and sclerosis. During long-term hemodialysis, cystic changes, so-called acquired cystic renal disease (ACRD) may develop. In spite of sclerosis, the kidneys in patients with ESRD/ACRD show remarkable mitotic activity. Intense cell proliferation is accompanied by pre-neoplastic lesions and unique types of renal cell carcinomas (RCC) develop at higher frequency than in the general population (3, 4). Clinically recognized RCC of end-stage kidneys has been found in 3.8% of prospectively screened candidates for renal transplantation, whereas the general population has a 0.04% lifetime risk of developing RCC (5, 6).

Several studies have suggested a role of a progressive inflammatory microenvironment in remodeling of ESRD/ACRD kidneys and the development of tumors (7-11). However, less is known about the role of 1,25-$(OH)_2D_3$ (vitamin D) metabolism in ESRD/ACRD-associated carcinogenesis. Vitamin D has strong antiproliferative and antitumorigenic effects and plays a role in the maintenance of normal cellular status quo by interaction with nuclear VDR (12-15). The level of circulating vitamin D is tightly regulated by the expression of two hydroxylases in epithelial cells in the kidney. 1α-Hydroxylase (cytochrome P450 family 27 subfamily B member 1, CYP27B1) is responsible for synthesis of the biologically active form of vitamin D, whereas 24-hydroxylase (CYP24A1) mediates its catabolism (16). CYP24A1 has a vitamin D response element in its promotor region and acts in control mechanisms to prevent tissue vitamin D intoxication (17).

Reduced CYP27B1 and increased CYP24A1 expression have been demonstrated in cancer cell lines, suggesting that dysfunctional vitamin D metabolism may be involved in carcinogenesis (18-20). The aim of this study was to analyze the possible role of vitamin D metabolism in remodeling of

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kidney tissue in ESRD/ACRD and in tumor development by comparing the expression to that of normal fetal and adult kidneys. We applied VDR, CYP24A1 and CYP27B1 immunohistochemistry to kidneys, ESRD/ACRD and associated renal cell tumors.

Patients and Methods

Tissue samples. We collected 12 kidneys removed from patients with ESRD/ACRD due to cancer. The formalin-fixed kidney specimens were obtained from the Departments of Urology at the University of Heidelberg, Bad-Hersfeld District Hospital, Germany, Radcliffe Hospital in Cambridge, United Kingdom and of the Institute of Pathology, University of Ljubljana, Slovenia. Six kidneys were classified as ESRD and six with intensive cystic changes as ACRD. Each kidney was processed entirely in paraffin blocks for histological analysis. Hematoxylin and eosin-stained slides were scored for cysts, small precursor lesions and tumors. The diagnosis of the main tumors was established according to the Heidelberg Classification and as proposed by Tickoo *et al*. (21, 22). Five tumors were diagnosed as papillary RCC and another six as conventional RCC. We also found one oncocytoma, two ACRD-associated eosinophilic-vacuolated tumors, two chromophobe-like tumors, and one clear-cell papillary RCC in the 12 kidneys. Altogether, 65 small papillary, 42 chromophobe-like and 24 eosinophilic vacuolated pre-cancerous lesions were detected in the 12 kidneys (10).

Tissue multiarray (TMA) containing 3-5 core biopsies from each tumor was constructed from paraffin-embedded tissues after marking the areas of interest on hematoxylin and eosin-stained slides by one of the Authors (GK). Core biopsies of 0.6 mm in diameter were placed within a recipient block by Manual Tissue Arrayer (MTA1; Beecher Instruments, Inc. Sun Prairie, WI, USA). Fetal kidneys were obtained from autopsy whereas adult kidney tissues were from radical tumor nephrectomy. The use of tissue samples for this study was approved by the Ethics Committee of the University Pecs, Hungary (no. 5343/2014). All procedures were in accordance with the ethical standards of the Institutional Research Committee and with the 1964 Helsinki Declaration.

Immunohistochemistry. Sixteen representative paraffin blocks from kidneys from patients with ESRD/ACRD were selected for immunohistochemistry. Serial sections were used in order to be able to compare the results of immunohistochemistry. We also analyzed fetal and adult kidneys for the cellular localization of the antibodies. After dewaxing and rehydration of the slides, antigen de-masking was performed in 10 mM sodium citrate buffer, pH 6,0 in 2100-Retriever (Pick-Cell Laboratories, Amsterdam, the Netherlands). Endogenous peroxidase activity and nonspecific binding sites were blocked with 3% hydrogen peroxide containing 1% normal horse serum for 10 minutes at room temperature. Slides were then incubated with antibodies to VDR (ab134826, 1:200 dilution; Abcam, Cambridge, UK), CYP27B1 (EPR20271, 1:2000 dilution; Abcam) and CYP24A1 (HPA022261,1:200 dilution; Sigma-Aldrich, St-Louis, MO, USA) at room temperature for 1 hour each. Horse radish peroxidase-conjugated secondary antibody (Histopathologia Kft, Pecs, Hungary) was applied for 30 minutes. The signal was then visualized with amino-ethyl-carbazol (DAKO, Glostrup, Denmark). Tissue sections were counterstained with Mayer's hematoxylin (Lillie's modification, DAKO) and after 10 seconds bluing slides were mounted by Glycergel Mounting Medium (DAKO). For negative controls, the primary antibody was omitted. The staining intensity was assessed by comparing it to the intensity observed in adult normal kidneys.

Results

Expression pattern of VDR, CYP27B1 and CYP24A1 in fetal and adult kidneys. We found strong expression of VDR in the nephrogenic zone of fetal kidney (Figure 1A). Cells of the tip of the ureteric bud, cap of the metanephric mesenchyme, pretubular aggregates, renal vesicles, comma-shaped body and the S-shaped body displayed strong nuclear staining with VDR antibody in 10 gestational-week-old kidneys. The strongest expression was seen in the distal compartment of S-shaped body. In 14- and 16-week-old fetal kidney proximal and distal tubular cells also displayed weakly positive staining with VDR antibody. CYP27B1 protein was strongly expressed in cells of emerging Henle loops, macula dense cells and distal tubules in 10-, 14- and 16-week-old kidney (Figure 1C). No expression was detected by the CYP24A1 antibody in fetal kidneys.

In adult kidney, nuclear VDR expression was observed in all types of cells from the proximal tubules to medullary collecting tubules, but the strongest expression was seen in distal tubules (Figure 1B). CYP27B1 exhibited strong expression in the ascending loop of Henle, macula dense cells and distal tubules (Figure 1D). CYP24A1 expression in distal tubules was weak. Both CYP27B1 and CYP24A1 proteins were present in the cytoplasm of cells, with fine granular staining corresponding to their mitochondrial location.

Expression of VDR, CYP27B1 and CYP24A1 in kidney tissue from patients with ESRD/ACRD. The strong structural remodeling of kidneys in patients with ESRD/ACRD was characterized by highly fibrotic inflammatory stroma containing small cell clusters, circumscribed proliferation of slightly dilated tubules and different types of cysts, as well as areas displaying atrophic thyroid-like structure. Therefore, positive immune reaction cannot be precisely localized to segment-specific cells of the renal tubular system. VDR, CYP27B1 and CYP24A1 expression was seen only in some solid cell clusters and in small clusters of growing tubular cells (Figure 1E-G). Within the thyroid-like area, occasional solid clusters of cells displayed VDR, CYP27B1 or CYP24A1 positivity. Altogether, only 1-3% of the epithelial cells displayed positive staining for each of VDR or CYP27B1 or CYP24A1. Some of the cysts were lined by positively stained cells and others by negatively stained cells. In three ACRD kidneys, several cysts with papillary-solid eosinophil-vacuolated cells growing into the lumen were detected, all showing positive staining for VDR and CYP27B1, and weakly for CYP24A1. Of interest, two kidneys obtained from the same patient with ESRD showed diffuse positive staining for

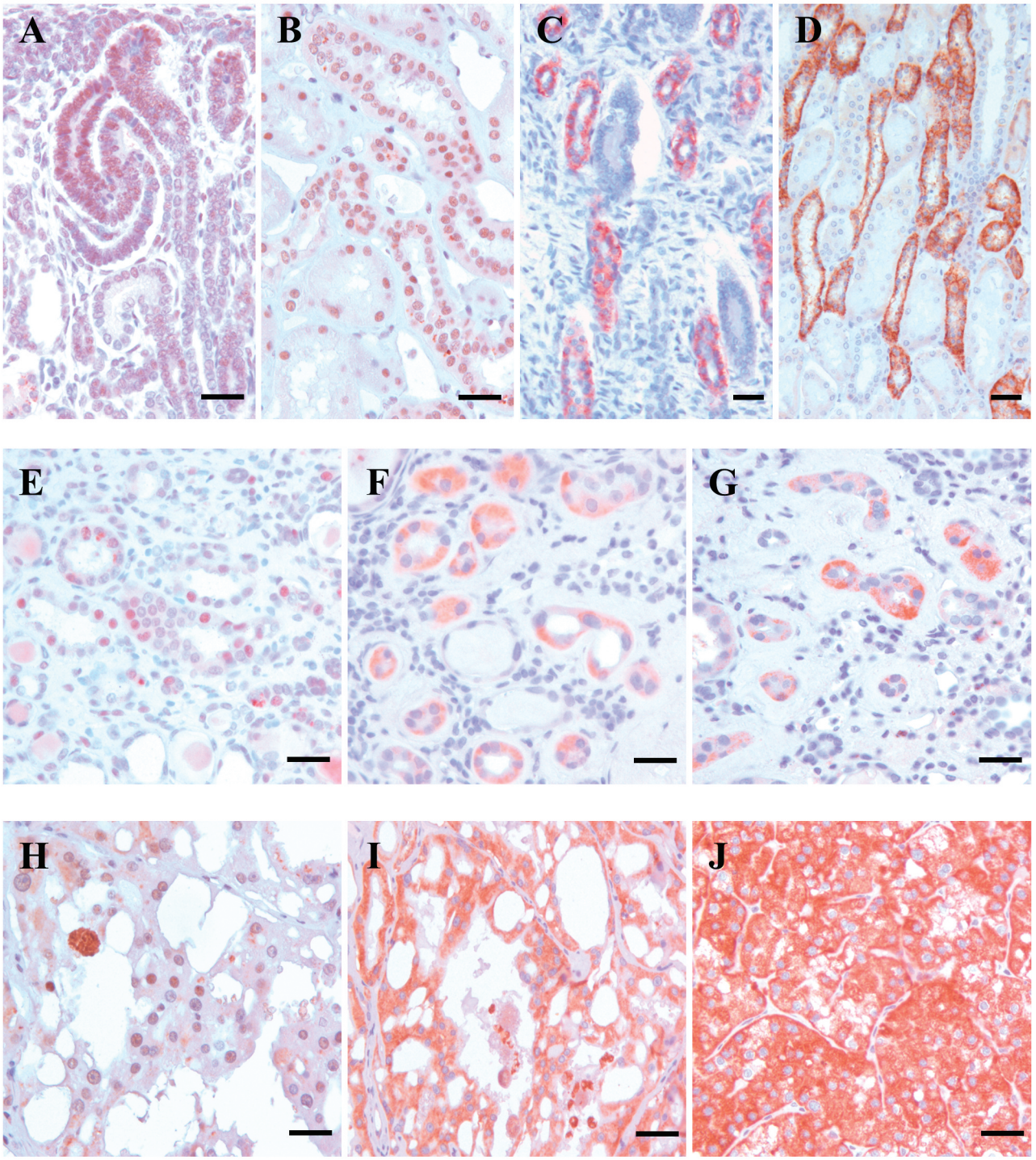

Figure 1. *Expression of vitamin D receptor (VDR), cytochrome P450 family 27 subfamily B member 1 (CYP27B1) and cytochrome P450 family 24 subfamily A member 1 (CYP24A1) in normal, and end-stage renal disease and acquired cystic renal disease (ESRD/ACRD) kidneys and tumors. Strong nuclear VDR expression was found in fetal kidney (A). Nuclear expression of VDR was found in all epithelial cells of adult nephron (B). Strong immunoreaction with CYP27B1 antibody was apparent in thick ascending loop of Henle and distal tubules in fetal (C) and adult (D) kidney. VDR (E), CYB27B1 (F) and CYP24A1 (G) staining of epithelial cell groups embedded in fibrotic stroma of ESRD kidney. Eosinophilic-vacuolated tumor showing nuclear VDR (H) and cytoplasmic CYP27B1 (I). Strong cytoplasmic immunoreaction of CYP27B1 was found in chromophobe-like renal cell carcinoma (J). Scale bar: 25 μm.*

VDR, CYP27B1 and CYP24A1 in approximately 30% of epithelial cells. Several small chromophobe-like pre-neoplastic lesions were also positive for VDR, CYP27B1 and CYP24A1.

Expression of VDR, CYP27B1 and CYP24A1 in ESRD/ACRD-associated tumors. Several small pre-neoplastic lesions were detected in the slides used for immunohistochemistry. Six papillary RCCs included in TMA as well as all papillary pre-neoplastic lesions were consequently negative with the three antibodies. No expression of VDR, CYP27B1 or CYP24A1 was observed in the six conventional RCCs and the clear-cell papillary RCC. Eosinophil-vacuolated RCCs and their precursors were positive for VDR and CYP27B1 (Figure 1H and I), and weakly for CYP24A1. All chromophobe-like tumors, as well as their precursor lesions displayed strong positive staining for CYP27B1 (Figure 1J) and moderate staining with antibodies against VDR and CYP24A1.

Discussion

Vitamin D is synthesized, presumably from maternal $25(OH)_2D_3$/vitamin D-binding protein complex, in distal tubules of fetal kidney as early as the 10^{th} week of pregnancy. By binding to VDR in the nephrogenic zone, it induces target gene expression and controls fetal nephron development. In adult kidney, VDR was found to be ubiquitously expressed in all cells of the nephron, whereas the expression of CYP27B1 and CYP24A1 was limited to the distal nephron. The coordinated expression of these three is necessary to keep the level of circulating vitamin D at an optimal physiological concentration and regulate mineral homeostasis.

In kidney from patients with ESRD/ACRD, a progressive inflammatory and fibrotic microenvironment replaces the functionally intact renal tubules. The remaining few epithelial cells of unknown origin make up approximately 5-10% of epithelial cells of that of normal kidney. Among these cell clusters, we found a substantially reduced number of CYP27B1-, VDR- and CYP24A1-expressing cells (1-5%), which contrasts with the 100% and approximately 40-50% of cells positive for VDR and CYP27B1, respectively, in normal kidneys. These observations may explain the low level of circulating vitamin D in patients with ESRD/ACRD and at the cellular level in affected kidneys.

Active forms of vitamin D have a strong anti-tumorigenic effect on precancerous and cancerous lesions (12-14). Several studies demonstrated reduction of CYP27B1 and increased CYP24A1 expression in breast, colon and prostate cancer cell lines, suggesting an association between dysfunctional vitamin D metabolism and carcinogenesis (18-20). We found in this study that ESRD/ACRD-associated conventional RCCs, clear-cell papillary RCC, papillary RCCs and their precursor lesions did not express VDR or CYP27B1, indicating that tumor cells had escaped vitamin D control. ESRD/ACRD-associated eosinophilic vacuolated tumors, chromophobe RCC-like tumors and their precursor lesions displayed strong expression of VDR and CYP27B1 and a weak expression of CYP24A1. It is noteworthy that these two types of renal tumors occur exclusively in ESRD/ACRD, whereas conventional RCC and papillary RCC also develop in the general population.

There is increasing evidence that in addition to mineral homeostasis, vitamin D may have other biological functions. Vitamin D plays a crucial role in maintenance of status quo of differentiated cells by controlling cell proliferation, apoptosis, angiogenesis, invasion and metastasis (23-26). Vitamin D has strong antiproliferative, pro-differentiation and pro-apoptotic actions in some cells, tissues and cancers (27, 28). A normal level of vitamin D reduces the risk of development of renal, colorectal, breast, lung and bladder cancer and lethal prostate cancer (29-31). The biological effect of vitamin D is mediated through its binding to VDR (27). It was shown that vitamin D–VDR complex inhibits cell proliferation by up-regulation of *p21* and *p27* (32). VDR also controls cell migration and invasion by increasing the expression of E-cadherin (33). VDR is involved in apoptosis by inhibiting the expression of anti-apoptotic proteins BCL2 apoptosis regulator (BCL2) and BCL2-like 1 (34).

Recently it was demonstrated that VDR suppresses the proliferation and metastatic potential of RCC cell lines *via* regulation of transient receptor potential cation channel subfamily V member 5 epithelial Ca^{2+} channels (35). Vitamin D inhibited growth of human kidney cancer cells and suppressed tumor growth *via* interaction with VDR (36, 37). The lack of a protective effect of vitamin D due to its diminished expression increases the risk of kidney cancer development (38). We showed in this study that CYP27B1 as well as VDR expression is substantially down-regulated in kidneys from patients with ESRD/ACRD, which may lead to a high risk of tumor development.

In conclusion, our study demonstrates a significant decrease in expression of CYP27B1 and VDR in ESRD/ACRD kidneys which may explain the low level of local and circulating active vitamin D. It was reported that a progressively pro-tumorigenic inflammatory microenvironment is associated with tumorigenesis (9). We showed in this study that the lack of antitumor effect of vitamin D signaling may also be a crucial factor in the frequent development of unique types of RCC in patients with ESRD/ACRD.

Conflicts of Interest

The Authors have no conflicts of interest to declare.

Authors' Contributions

JD and GK designed this study. JD and DB carried out the immunohistochemistry, JB and GK performed the evaluation of the

results. JD wrote the first draft of the article, and GK, TF and AS revised the article. All Authors read and approved the final version of the article for publication.

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