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Constitutive Expression of HIF- α Plays a Major Role in Generation of Clear Cell Phenotype in Human Primary and Metastatic Renal Carcinoma

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Abstract

The extensive lipid accumulation occurred in clear cell renal cell carcinoma (ccRCC) resulted in a clear cell cytoplasm. Hypoxia inducible factor alpha (HIF- α) was constitutively expressed in many ccRCC and transcriptionally regulates greater than one hundred genes. In a recent breakthrough study, HIF-1 α induced ccRCC in transgenic mice. Based on these findings, we developed a hypothesis that accounted for HIF- α generation of the clear cell phenotype. The aim of the present study to use immunohistochemical staining methods in tissue microarray (TMA) to determine the extent to which the clear cell phenotype coincided with HIF- α expression in primary and metastatic ccRCC. In addition, we studied whether the prolyl-hydroxylases (PHD2,3) play a role in promoting the elevated expression of HIF- α in tumor cells. The clear cell phenotype was observed in all primary and metastatic cases of ccRCC examined. A total of 168 renal cell carcinomas were evaluated by immunohistochemical methods; 141/168(84%) of tumors expressed HIF- α (HIF-1 α and/or HIF-2 α). In contrast, HIF- α was expressed in only 1/23 (4%) of non-clear cell renal cell carcinomas. These data supported the hypothesis that in the majority of the tumors HIF- α expression overlapped with the clear cell phenotype, and was indicative of a HIF- α mediated lipid accumulation. In a smaller percentage of ccRCC cases (16%) HIF- α was not detected in the tumor cells and suggested that lipid accumulation by HIF- α -lipid independent process. PHD3 was undetectable in both primary and metastatic ccRCC cases. We concluded that the undetectable PHD3 could contribute to the higher HIF- α expression in ccRCC.

Keywords

clear cell renal carcinoma; HIF- α ; PHD2,3; immunostaining

INTRODUCTION

Lipid accumulation in the cytoplasm of benign tumors (e.g, lipoma, cortical adenoma of adrenal gland) and malignant tumors (e.g., liposarcoma) is readily understandable based on the common knowledge that the cells from which they originate accumulate lipid as well. In these tumors, the lipid accumulating capability of the cytoplasm of the tumor cells remains even after the onset of malignant transformation. In other rare tumors, lipid storage in the cytoplasm is unusual and is more difficult to explain. A second case of a unique, rare lipid-rich cell, thyroid adenoma, including a detailed lipid analysis was previously described by Tóth, et al¹. This earlier paper demonstrates the use of HPLC to determine qualitative differences between triglyceride composition in tumor cells and subcutaneous fat. The unusual fat accumulation in the follicle cells results from an altered intracellular lipid metabolism rather than the result of a simple storage of lipids.

Clear cell renal cell carcinoma (ccRCC) is the most common cancer of the kidney in adults. The tumor cells exhibit a characteristic histological feature of clear cytoplasm because of the large amount of lipid accumulation in the cytoplasm.² This lipid consists principally of cholesterol esters. Glycogenesis is also regulated by HIF-1 α . Glycogen accumulation also contributes to the clear cell phenotype of the renal cell carcinoma cells, however, the current study focused solely on lipid accumulation by the ccRCC cells. This lipid is removed during the standard immunohistochemical processing, and results in the clear cytoplasm of the tumor cells. It is generally accepted that ccRCC originates from proximal tubular cells that do not contain excessive lipid in the cytoplasm and they are not clear cells cytologically. The reason for this well known, unusual lipid accumulation is not fully understood. It is suggested that this phenotype might be associated with the mutation of the von Hippel-Lindau (VHL) tumor suppressor gene resulting in a constitutive, permanent expression of hypoxia inducible factor-alpha (HIF-1 α and/or HIF-2 α) that transcriptionally regulates more than hundred genes reviewed by Semenza³. A recent breakthrough study demonstrated that mutant, constitutively active HIF-1 α induced ccRCC in transgenic mice⁴, and provides strong experimental evidence that HIF-1 α functions as an oncogene that is capable of inducing the clear cell phenotype of renal cancer in mice.

The aim of the present study was to use immunohistochemical methods to determine whether the clear cell phenotype coincided with HIF- α expression in human primary and metastatic (matched and unmatched) ccRCC. In addition, we studied the role of prolyl-hydroxylases (PHD2,3) crucial regulators of HIF- α in promoting the elevated expression of HIF- α in tumor cells.

MATERIALS AND METHODS

Human ccRCC Specimens

Tissue microarray (TMA) blocks were constructed from conventional paraffin blocks of ccRCC specimens. Surgical specimens were fixed in 10% buffered formalin embedded in paraffin. Each tumor was represented by one core (0.6mm in diameter); the cores were carefully selected by a board certified pathologist to ensure that 100% of the kidney cancer tumor cells possessed the characteristic clear cell phenotype. One TMA (GUCa2a)

contained 88 evaluable cores from 88 primary ccRCC cases. For comparative purposes, this TMA was complemented with 23 cores from 23 patients with primary non-clear cell renal carcinoma (e.g. papillary carcinoma, oncocytoma). The other TMA (GUCa4) contained 46 matched cores from 23 cases of primary and metastatic carcinoma from the same patient. This latter TMA contained an additional 61 unmatched metastatic ccRCC from 61 different patients. Cores from several matched and unmatched normal (tumor-free) kidneys were also included in the TMAs.

Immunohistochemical Staining for HIF-1 α , HIF-2 α , PHD2 and PHD3

Our laboratory developed, optimized and characterized a reliable double immunohistochemical staining methods for HIF-1 α and its regulatory molecules PHD2 and PHD3 in human tissues that were formalin-fixed and embedded in paraffin blocks.^{5,6} These assays can also be used to detect individual markers. The same basic procedure can be used to detect HIF-2 α , however, in this latter procedure the primary antibody must be against HIF-2 α lpha.⁷ Briefly, paraffin sections (5 μ m thickness) were cut from TMA blocks and immunostained separately with an automatic immunostainer for the individual markers. For HIF- α detection, it is important to use a Target Retrieval Solution (TRS-Dako Carpinteria, CA) that had been microwaved for 20 minutes. It is necessary to utilize a Catalyzed Signal Amplification (CSA) reagent during the immunohistochemical staining procedure to detect HIF- α localized in the nucleus. The following primary antibodies were used in the staining procedures: for HIF-1 α : mouse anti-human MAb, (clone H1 α 67) at 0.4 μ g/ml; for HIF-2 α : mouse anti-human MAb (clone ep 190b) at 0.5 μ g/ml. Both anti-HIF- α Mabs were obtained from Novus Biologicals, Littleton, Co. For PHD2: rabbit anti-human PAb also from Novus at 6 μ g/ml; for PHD3: rabbit anti-human PAb from Abcam, Cambridge, MA at 4 μ g/ml. All primary antibodies were incubated overnight at 4°C. In place of the primary antibodies, isotype- matched immunoglobulin controls were placed on duplicate slides (in the same concentration as the primary antibodies) as negative controls to verify the staining specificity.

Established positive control tissues for the various markers were incorporated into the tests to ensure that the actual methods were working properly. For HIF-1 α , a human head and neck squamous cell carcinoma xenograft was used in which the well differentiated portion of the tumor was avascular and, therefore, hypoxic. Our laboratory had previously demonstrated that the tumor cell nuclei in this area stained strongly positive for HIF-1 α .⁶ For HIF-2 α , nuclei of Kupffer cells of normal human liver served as a positive control; — liver cell nuclei are known to stain negative for HIF-2 α .⁸ Cytoplasm of the tubular epithelium of the normal human kidney were utilized as a known positive control for PHD2 and PHD3 as we reported.⁶ Normal human tissues under physiological conditions are not hypoxic, therefore HIF- α is undetectable by immunohistochemical methods.⁶

Semiquantitative Assessment of the Immunostaining

HIF-1 α and HIF-2 α immunostaining were considered specific if they were localized in the nuclei. For PHD2 and PHD3 the cytoplasmic immunostaining was evaluated as specific. Staining intensity in the core tissues were compared with the known strongly stained positive controls and categorized accordingly: strong (s), moderate (m) and weak (w) or not

detectable in any tumor cells. Since one core (0.6mm in diameter) of the TMAs contained approximately 600 tumor cells (varied based on the extent of stroma with vessels, inflammatory cell infiltration, necrosis, etc.) The estimated 1% of the total tumor cell population (in general 5-6 tumor cells at least) was considered as positive. A board certified pathologist (K.T) did the initial reading of the TMAs; the immunostaining intensity of the individual cores was estimated and categorized as weak (w), moderate (m) and strong (s), followed by a number that revealed the percentage (distribution) of tumor cells stained. For example: s/100 would represent a core in which 100% of the tumor cells stained strongly for a particular marker. The basic data were tabulated and presented in the Result section of the manuscript.

RESULTS

Incidence of HIF- α Expression and Renal Cell Carcinomas

Table 1 compares the incidence of HIF-1 α in the homogeneous clear cell phenotype of primary and metastatic renal cell carcinoma cores (cases) with that of a mixed group of primary renal cancers (e.g., papillary cancer, oncocytoma) without the clear cell phenotype. HIF- α (HIF-1 α and/or HIF-2 α) together is significantly higher in groups 1 and 2 with clear cell renal cancer than in group 3 that consists of non clear cell renal cell cancers. The combined average incidence of HIF- α expression in the primary and metastatic clear cell carcinoma group was 84% (141 of 168 cases) compared to a 4% incidence in the non clear cell carcinoma group (1/23 cases).

Table 2 compares the incidence of HIF- α expression between primary ccRCC and the matched metastases from the same patients. The objective of this study was to determine whether the metastatic process influenced the extent of HIF- α expression. Based on the limited number of matched cases (n=23), it appeared that the incidence of HIF-1 α expression in metastases was significantly increased compared to the HIF-1 α incidence observed in primary ccRCC.

Table 3 reveals the incidence of HIF-1 α and HIF-2 α expression in TMA of primary ccRCC (n=88) compared to all available matched (n=23) and unmatched (n=34) ccRCC metastases together (n=57). Data regarding the HIF- α expression in primary ccRCC without metastases has been published previously from our laboratory⁷. These data indicates that the incidence of HIF-1 α expression increased and HIF-2 α expression decreased significantly in the ccRCC metastatic group as compared to the primary ccRCC group. In most cases, the nuclear staining intensity was strong for HIF-1 α and HIF-2 α .

In Primary ccRCC HIF-1 α incidence alone was 9%, as compared to the much higher 46% incidence for HIF-2 α (Table 3). The distribution (i.e., the number of tumor cell nuclei that are positive in a given tumor) varied in the opposite direction: the average distribution for HIF-1 α and HIF-2 α were 66% and 15%, respectively.⁷ Forty six percent of the tumors tested expressed only HIF-2 α , but in the positive samples generally, few tumor cell nuclei (15%) expressed HIF-2 α . In the combined cases of metastatic ccRCC, the average distribution of HIF-1 α and HIF-2 α were 42% and 3%, respectively.

Immunohistochemical Staining of HIF- α and PHDs in Primary and Metastatic clear cell Renal Cell Carcinoma

Figure 1 consists of selected representative photomicrographs of individual immunohistochemical staining of HIF-1 α , HIF-2 α , PHD2 and PHD3 expression of primary and metastatic renal cell cancers (all original magnification at 400x) Panels 1 and 2 represented different primary ccRCC cases immunostained for HIF-1 α and HIF-2 α . In Panel 1, the arrow reveals strong, specific nuclear staining for HIF-1 α in many clear cells. In Panel 2, the arrow points to many fewer cells that express HIF-2. Panel 3 represents a papillary renal cell cancer without a clear cell phenotype and demonstrate no immunostaining for HIF-1 α . Panel 4 and 5 represent different metastatic ccRCC cases that were stained for HIF-1 α and HIF-2 α ; the frequency of staining was considerably less for HIF-2 α . Arrows show the specific nuclear staining. Panels 6 and 7 represent the same lymph node metastasis of a ccRCC. Consecutive sections were immunostained for PHD2 and PHD3, respectively. Panel 6 demonstrates that the cytoplasm of metastatic tumor cells was strongly immunostained for PH2 (as revealed by stars). No staining was observed in the population of lymphocytes (L). Panel 7 shows that the same tumor cells (stars) are negative for PHD3. The strong immunochemical staining of lymphocytes served as an internal positive control for the staining method, and demonstrated that the lack of immunoreactivity in the cytoplasm of tumor cells was not due to suboptimal immunostaining method for PHD3. In a previous paper that studies the distribution of PHDs in normal organs, Soilleux et al had demonstrated that PHD3 is expressed in lymphocytes.⁹ We determined that all primary and metastatic tumors failed to stain for PHD3. This observation is a novel finding in our laboratory concerning the molecular characterization of ccRCC. In contrast to this finding PHD3 was detected in 12 of 23 cases of non clear cell renal cell carcinoma in our current study and in 21% of human head and neck cancers and in 50% of colorectal cancers.⁷ Immunostaining incidence for PHD2 in primary ccRCC was 35%⁷ in the combined metastatic cases the incidence of PHD2 was 70%. The average distribution of PHD2 in primary and metastatic ccRCC was 64%⁶ and 56%, respectively. In general, the staining intensity for PHD2 was weak in primary tumors⁷, and moderate in metastatic ccRCC.

DISCUSSION

In the combined groups of all primary and metastatic ccRCC cases, 141/168 tumors expressed HIF- α (HIF-1 α and/or HIF-2 α) (84% incidence). In contrast, only 1/23 cases of non clear cell renal cell carcinoma group expressed HIF- α . (4% incidence). This marked overlap of nuclear HIF- α expression with the clear cell phenotype suggested that in the majority of cases, an association existed between a specific genotype nuclear HIF- α and clear cell cytoplasm phenotype. These data supported the hypothesis that a cause-effect relationship existed between HIF- α and the clear cell phenotype in human ccRCC.

Two new molecular mechanisms have been described recently^{2,10} that might explain our immunohistochemical findings concerning the high coincidence of nuclear HIF- α expression with the high cytoplasmic lipid content of clear cell renal cell cancers. A newly isolated very low-density lipoprotein receptor (VLDL-R), whose transcription is regulated by HIF-1 α was present in levels four-fold greater in ccRCC biopsies (n=6) than in normal

kidney. This receptor binds and mediates the uptake of triglyceride-rich lipoproteins by endocytosis and leads to the lipid accumulation in the cytoplasm of clear cell tumor cells. These data and additional detailed molecular studies reported in this paper support the concept that the pathological, constitutively increased expression of HIF-1 α in ccRCC cells upregulated the VLDL-R, which mediates the increased uptake and accumulation of lipids.²

The other recently delineated novel molecular mechanism of lipid accumulation in clear cells of renal cell carcinoma is linked to the hypoxia-inducible protein 2 (HIG2). This newly isolated lipid droplet protein is encoded by HIG2 gene which was shown as a specific target of HIF-1 α .¹⁰ Detailed, reliable molecular mechanistic studies have demonstrated that overexpression of HIG2 promotes an increased intracellular accumulation of neutral lipids. HIG2 is upregulated by hypoxia and HIF inducers in various cell types and is highly expressed in ccRCC. Only 1 out of 20 ccRCC was negative for HIG2 expression. In addition, HIG2 is a specific target gene of HIF-1 α (but is not responsive for HIF-2 α). The HIG2 promoter is directly transactivated by HIF-1 α . Immunofluorescence studies, revealed that HIG2 protein surrounds the lipid droplets. HIG2 is detected in atherosclerotic arteries and in fatty liver diseases as well. These observations suggest that this new HIF-1 α target gene might play an important role in other diseases associated with pathological lipid accumulation¹⁰.

Our studies showed that in 27 of 168 overall cases of ccRCC (16%) HIF- α was not detectable and the distribution of HIF-1 α and HIF-2 α was not homogeneous in several HIF- α positive tumors, and indicated the presence of a various number of tumor cell nuclei without immunostaining for HIF- α . We conclude that in HIF- α negative tumor cells with clear cytoplasm a HIF- α independent molecular mechanism could be responsible for the excessive lipid accumulation. This is not so surprising, as renal cancer is widely considered as a metabolic disease.¹¹ Although, false negative results for HIF- α cannot be completely excluded due to the technical limitation, such as inadequate sampling, which is a known risk of TMA approach.

We have previously reported that PHD3, one of the main regulatory enzyme of HIF- α expression, was not detected in any cases of ccRCC (n=88).⁷ In our current report, we extended our study to include metastatic ccRCC cases (n=61). Metastatic cells of ccRCC did not express either detectable levels of PHD3 when assayed by immunohistochemical method. The high incidence of HIF- α in ccRCC has been partially associated with the mutation of the VHL gene. The VHL mutation is not present in all cases of ccRCC; the overall incidence of this mutation varies from 19.6% to 89.4%^{12,13} The majority of the reports reveal a VHL mutation rate of 30-60%.¹⁴ In this paper, the upregulation of HIF-1 α and HIF-2 α were 88.2% and 100%, respectively. In contrast, the incidence of VHL mutation in these cases of ccRCC was 39.1%, which suggested the presence of a functional VHL- with HIF-1 α expression in many cases.¹⁴ It is well-documented and generally accepted that an overexpression of PHD3 decreases HIF- α expression while a lowered expression of PHD3 increases HIF- α expression. We concluded that the undetectable PHD3 expression in both primary and metastatic ccRCC could contribute to the higher HIF- α expression in ccRCC in a subgroup of cases which express the wild type VHL. In the majority of cases where VHL is not functional, this potential regulation cannot occur. It was an unexpected

finding that the overall incidence and expression level of PHD2 increased in metastatic cases as compared to primary ccRCC, despite the increased incidence of HIF- α expression in metastases. In the case of PHD2, the correlation with HIF- α expression was not clear and simple. It was demonstrated that PHD2 is involved in the regulation of HIF- α expression, but also plays an important role in angiogenesis as well.¹⁵ We assumed in the metastatic lesions (e.g lung, lymph node) the neoangiogenesis could differ from that of the kidney that could have influenced PHD2 expression and formation of metastatic loci.

HIF-1 α incidence increase, and HIF-2 α incidence decreased in metastases compared to primary ccRCC (Table 3). The greater incidence of HIF-1 α expression in metastatic ccRCC in both circumstances (matched in Table 2 and unmatched cases in Table 3) might be explained with the higher overall distribution of HIF-1 α (66%) in primary cancer⁷, what could increase the chance for metastasis. Lower incidence of HIF-2 α in metastatic ccRCC (Table 3) might resulted in a much lower overall distribution of HIF-2 α (15%) in primary ccRCC⁷, what could decrease the chance for metastases. The greater expression of HIF-1 α in metastatic foci than in primary ccRCC is in agreement with a previous study in which levels of HIF-1 α were higher in metastatic than in primary ccRCC.¹⁶ The roles of HIF-1 α and HIF-2 α are not clearly distinct in ccRCC. In a previous transgenic study, stable expression of HIF-1 α , but not HIF-2 α , induced ccRCC in mice.⁴ The new lipid droplet protein HIG2 is a specific target gene for HIF-1 α , but not HIF-2 α .¹⁰ In our recent study, the average distribution for HIF-1 α expression was 42% compared to a 3% distribution for HIF-2 α in metastatic ccRCC. Furthermore, HIF-1 α proved to be a prognostic marker for ccRCC.¹⁶ Based on these current findings, it appears that HIF-1 α is more important than HIF-2 α in ccRCC, although HIF-2 α is co-expressed frequently with HIF-1 α and suggests its potential influence in ccRCC. In a recent xenograft study with human RCC cell lines, loss of HIF-2 α expression impaired tumor growth, whereas loss of HIF-1 α expression increased tumor growth.¹⁷ It is difficult to explain the differential roles of HIF-1 α and HIF-2 α on tumor growth based on current knowledge. In the previous study¹⁷, the majority of the xenografts demonstrated sarcomatoid dedifferentiation, only one tumor (vector transfection) was clear cell RCC histologically. Sarcomatoid dedifferentiation is only a subgroup and does not represent histologically the most common and typical renal cancer because it does not have clear cytoplasm. The findings are true for this particular xenograft. We consider these data rather exceptional than characteristic. In the transgenic mouse model HIF-1, but not HIF-2 α induced clear cell RCC.⁴ Under different circumstances, the HIF- α isoforms can function differently in various cancers.

Figure 2 summarizes our hypothesis about the HIF- α mediated lipid accumulation and development of clear cell phenotype in renal cancer that combines the findings of our immunohistochemical studies with two recent molecular studies.^{2,10} Our studies showed that HIF- α is expressed in the majority of (81%) metastatic ccRCC cases as well. Since HIF- α is constitutively, permanently expressed in ccRCC and it does not change like hypoxia induced HIF- α . The results of transgenic studies suggest that HIF-1 α expression is the driving force of the tumor.⁴ Thus, HIF- α could be an influential target for the chemotherapy of metastatic ccRCC. This notion is supported by the important finding that mutations in the human tumor suppressor gene (VHL) in the murine transgenic models induced vascular tumors in the liver¹⁸, kidney¹⁹ or induced renal cysts²⁰, but failed to induce ccRCC tumors.

Confirmation of the hypothesis regarding the HIF- α mediated lipid accumulation in ccRCC (Fig.2) needs additional validation studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Representative photomicrographs of sections from TMAs of primary and metastatic clear cell renal cell carcinoma (ccRCC) immunostained individually for HIF- α and PHDs (all original magnification $\times 400$). Panel 1 and 2 are different primary ccRCC cases immunostained for HIF-1 α and HIF-2 α , respectively. Specific, strong nuclear staining (brown) for HIF-1 α and for HIF-2 α is shown by arrows in Panel 1 and 2, respectively. Panel 3 is a papillary renal cell cancer without clear cell phenotype in cytoplasm and HIF-1 α immunostaining in the nuclei. Panel 4 and 5 are different metastatic ccRCC cases for HIF-1 α and HIF-2 α detection, respectively. Arrows show several strong, specific, nuclear staining (brown) for HIF-1 α and limited staining for HIF-2 α . Panel 6 and 7 are the same case of lymphnode metastasis of ccRCC. Consecutive sections were immunostained for PHD2 and PHD3, respectively. Panel 6 demonstrated that the cytoplasm of metastatic tumor cells is immunostained strongly (brown) for PHD2 indicated by stars, while the lymphocytes (L) were unreactive. Panel 7 clearly shows that the same tumor cells are negative for PHD3 (stars) while the lymphocytes (L) exhibit strong immunostaining for the same marker, what can be considered as an internal positive control for the PHD3 staining.

Figure 2.

Proposed hypothesis of HIF- α mediated lipid accumulation and clear cell phenotype in renal cell carcinoma. VHL mutations and undetectable PHD3 could lead to constitutive overexpression of HIF-1 α /HIF-2 α . Primarily, the overexpression of HIF-1 α transcriptionally upregulates the lipid receptor (VLDL-R) and the lipid droplet protein (HIG2) promotes the increased lipid accumulation and results in the clear cell phenotype.