CAIX Immunostaining in Non-neoplastic Renal Diseases

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Abstract. Background/Aim: Carbonic anhydrase 9 (CAIX) is a transmembrane metalloenzyme that regulates cellular adhesion, proliferation, and intra/extracellular pH. It is expressed primarily through a hypoxia-inducible factor 1 (HIF-1)-dependent mechanism. Its over-expression is closely related to somatic mutations in the Von Hippel-Lindau (VHL) gene. Studies have shown that it is over-expressed in renal cell carcinoma. In this study, we aimed to assess the value of CAIX immunostaining as an ancillary diagnostic tool in renal malignancies and medical renal diseases. Patients and Methods: Slides of kidney tumors and medical kidney diseases were selected to evaluate CAIX expression. Intensity and staining patterns of CAIX were independently assessed by two pathologists. Results: Our results showed strong and diffuse box-like membranous staining pattern in the majority of the clear cell renal cell carcinoma (ccRCC) cases (47/59 cases; 94%). A strong, diffuse cup-shaped staining pattern was observed in clear cell papillary RCC. Variable positivity was observed in other RCC (renal cell carcinoma) subtypes. In non-neoplastic renal conditions, the majority of the cases were negative for CAIX, and only a few cases demonstrated patchy non-specific staining. Of note, a single case of transplanted kidney biopsy taken because of delayed graft function showed a focal area of dilated tubules lined by cells with clear cytoplasm and enlarged nuclei with prominent nucleoli. This area showed diffuse membranous staining for CAIX. Two cases of end-stage renal disease showed a focal circumferential membranous staining pattern for CAIX in dilated tubules. Conclusion: The CAIX immunoreactivity observed in these three cases could be indicative of an early-stage renal cell neoplasm and warrants further investigation.

Carbonic anhydrase 9 (CAIX) is a transmembrane metalloenzyme that catalyzes the reversible hydrolysis of carbon dioxide (1, 2). The involvement of CAIX in regulating intra and extracellular pH, adhesion, and proliferation has been investigated extensively, and several studies have elucidated the role of CAIX in promoting tumor growth and metastasis (2-5). High expression of CAIX is mediated through hypoxia-inducible factor 1 (HIF-1) dependent mechanisms closely related to the development of Von Hippel-Lindau (VHL) defective tumors (2, 6). These HIF-1 mediated mechanisms allow drastic plasticity in gene expression that in turn, creates a conducive micro-environment for tumor growth and metastasis. The combination of low oxygen tension, high hydrostatic pressure, and an acidic extracellular pH helps tumors meet their elevated bioenergetic needs and promote angiogenesis and metastasis (7, 8). Besides hypoxia, other factors and molecules such as lactate, succinate, and reactive oxygen species can activate HIF-1 and subsequently CAIX (7).

CAIX is normally expressed in gastric mucosa, duodenal mucosa, and bile duct. The use of CAIX as a diagnostic marker for ccRCC is well-established, with a reported 85-100% sensitivity, while showing no significant staining in other RCC subtypes (2-4, 6). A few studies have indicated that high levels of CAIX expression are limited to a very few non-neoplastic conditions (2).

To date, most studies have focused on the evaluation of CAIX staining in RCC compared to normal kidney tissues or comparing its staining among RCC subtypes. However, as CAIX immunostaining becomes a standard in the diagnosis of ccRCC, it is prudent to evaluate whether CAIX immunostaining is specific for ccRCC, or whether it is also...
observed in hypoxic environments of other medical renal diseases. Normal kidney parenchyma showed absent or focal weak non-specific staining (1, 9, 10). One study showed no significant CAIX expression in 35 cases that were either normal kidney parenchyma, or benign kidney lesions such as pyelonephritis, renal cysts, adenoma, and oncocytoma (2).

In addition to benign and malignant tumors, there are a number of non-neoplastic kidney conditions that are thought to be characterized by variable degrees of renal hypoxia and fibrosis, so called “chronic hypoxia hypothesis”) (11, 12). Since CAIX expression is triggered by hypoxia, it is pertinent to investigate whether non-neoplastic chronic renal diseases thought to be associated with hypoxia also demonstrate staining patterns similar to those seen in renal cancers. Some of these renal conditions are predisposing factors for the development of kidney cancer. Some of these non-neoplastic conditions are also associated with morphological changes that could mimic ccRCC or some of the newly recognized subtypes of RCC. The identification of a focal cancerous component in these cases can, therefore, present a diagnostic challenge.

The purpose of this study was to investigate CAIX expression by immunohistochemistry in a wide array of non-malignant kidney conditions in comparison to RCC. The immunoreactivity, intensity and staining pattern were analyzed in order to determine whether CAIX is a sensitive and specific enough marker to differentiate neoplastic and non-neoplastic conditions.

### Patients and Methods

Tissue samples were collected from 164 patients. There were 60 resection specimens with ccRCC, 13 with each of papillary, clear cell papillary and chromophobe RCC. Sixty-five specimens (biopsies and resections) had non-neoplastic kidney disease including pauci-immune crescentic necrotizing glomerulonephritis (10 cases), acute antibody mediated rejection (10 cases), acute T-cell mediated rejection (5 cases), acute tubular injury (15 cases), interstitial nephritis (5 cases), end-stage renal disease (10 cases), and polycystic kidney disease (10 cases).

All samples were retrieved from the St. Michael’s Hospital archive after obtaining approval from the Research Ethics Board. All of the specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were cut 3 μm thick and stained with hematoxylin and eosin (H&E) to confirm the histopathologic diagnoses. All cases were immunostained for CAIX (Leica Biosystems, Carbonic Anhydrase IX, clone TH22, Concord, Ontario, Canada). CAIX expression was evaluated for staining pattern (cytoplasmic vs. membranous staining) and staining intensity (graded as mild, moderate, or strong). All slides were independently reviewed by two pathologists (SSK, AK).

### Results

#### CAIX expression in non-neoplastic renal conditions.

Immunohistochemical results of CAIX in non-neoplastic lesions were assessed in the following conditions: pauci-immune glomerulonephritis (N=10), antibody mediated rejection (N=10), T-cell mediated rejection (N=5), acute tubular necrosis (N=15), interstitial nephritis (N=5), end-stage renal disease (N=10), and polycystic kidney disease (N=10). A summary of the CAIX staining pattern is shown in Table I. Overall, either a negative or non-specific staining pattern was observed in all non-neoplastic cases with no consistent staining pattern among these conditions. The majority of chronic renal diseases exhibited fine granular, weak to moderate cytoplasmic staining for CAIX in some tubules and glomerular cells. Weak, patchy cytoplasmic tubular epithelial cell staining was observed in the cases of interstitial nephritis (Figure 1A), pauci-immune crescentic glomerulonephritis (Figure 1F), end-stage renal disease (Figure 1E), and polycystic kidney disease. Moderate levels of cytoplasmic staining were observed in acute tubular injury and acute rejection cases (Figure 1D). Rare membranous staining of tubular cells was observed in combined acute T-cell and antibody mediated rejection and end stage renal disease. In these cases, weak positive membranous staining of tubular cells was limited to single cells or a small cluster of cells (Figure 1B and C).
A biopsy of transplanted kidney 6 days post implantation showed acute rejection with an incidental finding of dilated tubules lined by cells with clear and eosinophilic cytoplasm, nuclear enlargement, and prominent nucleoli (Figure 2). These cells were diffusely strongly positive for CAIX in membranous pattern. These findings raise the possibility of a renal neoplasm, either an early neoplastic change (incipient neoplasm) or a biopsy adjacent to a neoplasm. Further imaging of the donor kidney did not demonstrate any renal masses. Because of the unusual CAIX staining pattern, the patient was followed closely for the possibility of developing renal neoplasm. Computed tomography (CT) scan of the
abdomen four years after transplantation showed transplanted kidney without any masses.

Two cases of end-stage renal disease showed foci of strong circumferential membranous staining for CAIX in tubular epithelial cells. On H&E slides, foci that expressed higher levels of CAIX showed dilated tubules/cystic architecture. Cells were enlarged with abundant clear cytoplasm. In contrast, the adjacent tissue that was negative for CAIX did not show any of these changes. The two cases were defined as “focal area suggestive of renal cell neoplasm and follow up was recommended”. These cases may therefore need to be further investigated to assess for the risk/presence of a renal cell neoplasm such as clear cell renal cell carcinoma or multilocular cystic neoplasm of low malignant potential. **CAIX expression in kidney cancers.** A strong diffuse box-like membranous CAIX staining pattern was observed in 56 out of 60 cases (93%) of ccRCC that were analyzed, in keeping with a previous report (13). Cases of clear cell papillary RCC were all positive for CAIX (100%) with a characteristic diffuse strong cup-shaped pattern (sparing the luminal border) (Figure 3). Chromophobe RCC was negative for CAIX. Papillary RCC showed mild to moderate focal positivity in 3 cases (30%). Staining in adjacent non-neoplastic kidney parenchyma was negative or non-specific.

**Discussion**

The results of our study confirm that a diffuse, strong, membranous CAIX staining pattern in a neoplastic kidney lesion is specific to ccRCC. Our results also suggest that finding foci of diffuse complete membranous staining of CAIX in dilated tubules lined by cells with clear cytoplasm should warrant further clinical/radiological investigations and follow up.
As biopsy specimens are increasingly used for the diagnosis of RCC and diagnosis may be compromised by suboptimal diagnostic material or co-existence of an underlying medical-renal condition, CAIX immunostaining can highlight the lesions of interest and help pathologists in reaching a diagnosis.

Using reverse transcription-polymerase chain reaction (RT-PCR), Li et al showed that CAIX expression was 100% specific for pathology-proven RCC and 68% sensitive (14). The lower sensitivity could be attributed to the fact that the authors analyzed multiple subtypes of RCC that we now know do not express CAIX equally. Our study confirmed similar findings using immunohistochemistry, which is more practical and easier to perform. We were able to demonstrate that a strong diffuse membranous CAIX staining pattern is observed almost exclusively in malignant cells. In fine needle aspiration cytology from renal fluids or kidney lesions with cystic contents, particularly in cases with image-indeterminate solid renal tumors, we could extrapolate that a positive diffuse staining pattern for CAIX in a fixed cytology specimen could indicate the presence of a malignant component (14).

In our study, we were able to identify additional smaller nests and individual malignant cells outside of the main tumor mass by using CAIX immunostaining. CAIX immunostaining can be of great value in assessing invasion of fat or identifying small clusters of neoplastic cells that may co-exist with inflammatory cells or cystic lesions resulting from end-stage renal disease/long-term hemodialysis (15, 16).

Our study has further value in delineating whether a cystic renal mass is benign or malignant in nature. Our results suggest that the presence of strong diffuse membranous staining in a cystic mass is strongly indicative of malignancy. A previous study comparing CAIX expression in cystic fluid from 16 cystic forms of RCC versus 12 benign renal cysts using ELISA showed high levels of CAIX expression in the malignant samples. In contrast, 11 of the 12 benign cystic tumors were negative for CAIX expression (17). Another study (18) examined 13 cystic ccRCC cases that all showed strong diffuse membranous staining for CAIX. Quantification CAIX protein in fluid from renal cysts using ELISA, showed a significant difference in the mean concentration of CAIX between malignant cystic tumors (mean expression level=2.043 pg/ml) and benign cysts (mean expression level=0 pg/ml). The same study showed that CAIX expression was concordant in almost 90% of the cases when measured using ELISA versus immunohistochemistry. This substantiates the validity of CAIX as a powerful diagnostic tool in differentiating between neoplastic and non-neoplastic conditions.

Few studies have suggested that CAIX is expressed in the pre-neoplastic cystic lesions and in the renal epithelial cells with malignant transformation (2). However, Hosseini et al., demonstrated that pre-neoplastic cystic lesions adjacent to nests of RCC revealed immunophenotypes similar to corresponding renal tumors in cases of acquired cystic disease (19). These findings suggest that a CAIX immunostaining could be used as a screening tool of malignant lesions in biopsied specimens. If not solely diagnostic, CAIX expression could still be a red flag to raise suspicion of malignancy and suggest closer follow up of cases that are at a high risk of developing RCC.

CAIX can help distinguish between subtypes of RCC. For example, a diffuse box-like membranous staining pattern is typically observed in cases of ccRCC, whereas a focal cup-like staining pattern (sparing luminal border) is typically observed in cases of clear cell papillary RCC (1, 10, 13, 15). Perinuclear dot-like immunopositivity can be also noted in type 2 papillary RCC (20). CAIX staining may also be used to differentiate between chromophobe RCC and ccRCC, as the former does not show specific staining pattern for CAIX. Similarly, a negative/non-specific staining pattern for CAIX in oncocytoma and translocation renal cell carcinoma has also been previously documented (1, 13, 21).

A diffuse, box like staining pattern for CAIX has also been documented in a plethora of other neoplastic conditions including malignant mesothelioma, adenomatoid tumors, endolymphatic sac tumors, epididymal papillary cystadenoma, clear cell adenosarcoma of the urothelial tract, pancreatic serous cystadenoma, and intrahepatic cholangiocarcinoma (1, 10, 22-25). Membranous CAIX positivity has also been noted in cases of intrahepatic cholangiocarcinoma, endocervical adenocarcinoma, pancreas carcinoma, and adrenocortical carcinoma (1, 10). Although other tumors such as urothelial carcinoma, endocervical adenocarcinoma, pancreatic adenocarcinoma, squamous cell carcinoma, gastric adenocarcinoma, colonic adenocarcinoma, and endometrial
endometrioid adenocarcinoma have also shown positive staining for CAIX, staining in these cases is less diffuse and often concentrated to areas of necrosis (1, 10, 26).

Further, the results of our study confirm earlier reports that show that CAIX could be a useful marker, either alone or in combination with other biomarkers, for the molecular subtyping of renal cancers. There is a rising trend of using molecular markers for molecular based sub-classification of renal cell carcinoma that replaces, or at least guides morphology-based diagnosis (27-29).

Additionally, diffuse membranous staining of CAIX could serve as a differential biomarker to point out to a renal origin of a metastatic cancer when the cells are poorly differentiated, and the original tumor cannot be determined.

Apart from kidney cancer, previous studies have also noted the potential diagnostic utility of CAIX in differentiating between different meningioma subtypes, as clear cell meningioma is negative for CAIX whereas microcystic and angiomatous meningioma stain positive (30). Recent reports have also suggested that in addition to being a diagnostic tool, CAIX can also serve as a prognostic marker and as a potential therapeutic target for RCC (5, 31). There is scarcity of prognostic markers for RCC that pathologists can use in their daily practice. One of the latest markers that is being investigated is DNA polymerase delta 1 catalytic subunit (POLD1), which is linked to the utility of CAIX immunostaining as a diagnostic tool to improved survival in patients with ccRCC (32).

In conclusion, we assessed CAIX expression in a spectrum of non-neoplastic renal diseases associated with hypoxia. A weak, non-specific cytoplasmic staining pattern was observed in the tubules and glomeruli of the non-neoplastic kidney cases assessed, whereas 94% of ccRCC cases assessed showed a specific, strong, diffuse, box-like membranous CAIX staining pattern. These results validate the utility of CAIX immunostaining as a diagnostic tool to differentiate between RCC and non-neoplastic kidney diseases, or to diagnose an incidental or hidden malignancy in the background of inflammation and cystic diseases.

Conflicts of Interest

All Authors have no conflicts of interest to declare in relation to this study.

Authors’ Contributions

AY and SSK – implemented study, collected/analyzed data, drafted and revised manuscript. AK – initiated project, designed data acquisition/collection tool, supervised data analysis, revised drafted manuscript. All Authors approved the final article.

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