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Redox State and Carbonic Anhydrase Isozyme IX Expression in Human Renal Cell Carcinoma: Biochemical and Morphological Investigations

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Clear renal cell carcinomas (RCC) frequently express carbonic anydrase IX (CA IX) because of non-functional mutation of von Hippel Lindau (VHL) tumor suppressor gene. CA IX is a tumor-associated transmembrane antigen, which catalyzes the extracellular, reversible hydration of carbon dioxide to bicarbonate and proton and thereby contributes to acidification of extracellular milieu. Extracellular acidic pH facilitates tumor growth and progression. CA IX expression is upregulated by Hypoxia Inducible Factor-1 (HIF-1), which is negatively controlled by oxygen via wild type VHL protein and is also regulated by the cell redox state. We investigated the immunohistochemical pattern of distribution of CA IX in a small series (14 cases) of RCCs. CA IX expression was matched with the redox state of RCC, stratifying our series in relation to clinical and histopathological parameters, such as Fuhrman grade, staging, proliferation markers expression, and particularly, the presence of necrosis. Our results show for the first time the existence of a perivascular pattern of CA IX distribution in RCC. We also found a significant relationship between CA IX expression and the presence of necrosis. Tumors with higher CA IX expression exhibited higher degree of necrosis ($p < 0.05$). Notably, an almost significant relationship between the redox state and CA IX expression was detected in RCC patients with 5 years disease-free survival, most of them showing organ-confined disease. Tumors with lower redox state showed an algebraically higher degree of CA IX expression. On the contrary, tumors with higher redox state exhibited an algebraically lower CA IX expression ($p = 0.057$). The observed relationship of CA IX expression and necrosis suggests a role for CA IX in RCC. Further investigations are necessary to further establish the role of the redox state in regulation of CA IX expression in RCC.

Keywords: Carbonic anhydrase; Clear renal cell carcinoma; Necrosis; Redox state; L-Glutathione

INTRODUCTION

Clear renal cell carcinomas (RCCs) frequently express a tumor associated transmembrane antigen, actually referred to as carbonic anhydrase IX (CA IX).[1] CA IX belongs to the α-CA gene family which is constituted of at least 14 members, 11 of them exerting catalytic activity.[2] CA IX catalyzes the extracellular, reversible hydration of carbon dioxide to bicarbonate and a proton. Through extracellular acidification and maintenance of normal intracellular pH, CA IX may contribute to the tumor growth and progression.[3,4]

CA IX expression is strongly activated by hypoxia-inducible factor HIF-1 in response to hypoxia.[5–9] Under normoxia, CA IX expression is negatively regulated by the wild type von Hippel Lindau (VHL) tumor suppressor protein that targets the α-subunit of HIF-1 to proteasomal degradation.[3,10] Non-functional mutation of VHL gene has been found in at least 60% of clear RCCs[11] and is associated

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with oxygen-independent CA IX expression.[5,9] HIF-1, the key regulator of CA IX expression, is also controlled by the cell redox state.[12] In fact, a protective role for HIF-1 in response to redox manipulation and glucose deprivation has been demonstrated.[13] Furthermore, HIF-1 is a target for S-nitrosylation by exogenously and endogenously produced NO.[14] Finally, NO, other than modulating the HIF-1 response under hypoxic conditions, also functions as a HIF-1 inducer.[12] Thus, through HIF-1, CA IX may respond to oxidative stress. Moreover, a common morphological feature of RCC is necrosis, which is strongly related to poor prognosis.[15] In fact, the true neoplastic necrosis, "dirty necrosis", represents the morphological counterpart of oxidative stress[16] and the distribution pattern of CA IX seems to be reinforced in perinecrotic areas in many tumours,[5] suggesting possible relationship between CA IX expression and oxidative stress. The importance of the oxidative stress in carcinogenesis has been underlined by Toyokuni,[16] who proposed a regulatory role in tumor growth and progression including genomic instability and angiogenesis. In the past, we found relevant variations in the glutathione antioxidant system in the different stages of renal cell carcinoma (RCC) and suggested an important role of oxidative stress in RCC growth and progression.[17] L-Glutatione is detectable in tissues in two different forms: reduced L-Glutatione (GSH) and oxidized L-Glutathione (GSSG). The GSH/GSSG ratio is related to the local redox state of the tissue. The primary biological function of glutathione (γ-Glu-Cys-Gly) is to act as a non-enzymatic reducing agent, which maintains cysteine thiol side-chains in a reduced state on the surface of proteins. Glutathione is also used to prevent oxidative stress in most cells, and helps to trap free radicals, which can damage DNA and RNA. The ability of cells to detoxify free radicals decreases when glutathione levels drop. Carbonic anhydrases, among which is CA IX, catalyze the reversible hydration of carbon dioxide, also generating protons.

In this contribution, our aim was to correlate the redox state of a series of RCC with the distribution pattern of CA IX, stratifying our series in relation to clinical and histopathological parameters such as Fuhrman nuclear grade, staging, proliferation markers expression, and particularly, the presence of necrosis.

MATERIALS AND METHODS

Selection of Cases and Histopathology

The tumor tissues consisted of 14 conventional RCCs selected from the files of the frozen tissue bank of the Institute of Pathology, University.of Siena (Italy). They were collected from patients who underwent radical nephrectomy for RCC during 1995. The specimens were immediately examined by a pathologist who collected several fragments of neoplastic tissue and the apparently normal renal cortex at a distance from the tumor. Necrotic and/or hemorrhagic areas were discarded and only yellow, firm tissue, mainly at the periphery of the tumor, was sampled. The material was immediately frozen in liquid nitrogen and stored at −70°C until used for the biochemical analyses. The remaining tissue was formalin-fixed and routinely processed. Histopathological diagnosis and classification of RCC was made according to the current diagnostic criteria. Grading was performed independently by two experienced pathologists according to the Fuhrman grade. Staging was done according to WHO criteria (TNM 1997). The whole tumor area of the slides was examined and the tissues were evaluated by 0 when necrosis was completely absent and by 1 in RCC with at least 1% of necrosis. Degenerative changes such as hyalinization or hemorrhage were not evaluated.

Immunohistochemical Analysis

Monoclonal antibody (M75) recognizing the N-terminal domain of MN/CAIX protein was employed to detect CA IX in RCC specimens.[18] Carbonic anhydrase isoenzyme CA IX was visualized using the avidin-biotin peroxidase complex (ABC) system, with reagents from Vector Laboratories (Burlingame, CA, USA). Paraffin sections (5 micron thick) were rehydrated through a series of graded ethanols and immersed for 30 min at room temperature in 0.3% H_2O_2 in methanol for inactivation of endogenous peroxidase. Blocking of endogenous avidin-binding activity was performed using an avidin-biotin blocking kit (Vector Laboratories). After washing (3 × 5 min) in 0.05 M phosphate-buffered saline (PBS), pH 7.6, sections were pre-incubated for 20 min in normal goat serum and diluted 1:5 with 1% bovine serum albumin (BSA, Sigma Chemical Co., St Louis, MO, USA) in PBS. This was followed by incubation with the monoclonal anti CA IX (1:100 in PBS added with 1% BSA) overnight at room temperature. After washing in PBS, biotinylated goat anti-mouse IgG (1:200) was used as a secondary antibody, for 45 min, followed by washing in PBS as before. Thereafter, sections were treated with the avidin-biotinylated peroxidase complex (ABC) (1:100) for 45 min and washed in PBS. The peroxidase activity was revealed using 3,3′-diaminobenzidine (DAB substrate kit, Vector Laboratories). The slides were counterstained with Carazzi's haematoxylin, dehydrated and mounted with Eukitt. Control sections, incubated with PBS instead of primary antibody, were unstained.

The evaluation of PCNA expression was performed by immunohistochemistry using the streptavidin-biotin method as previously described[17] and anti-PCNA antibody from DAKO ITALY.

Immunohistochemical Evaluation

All samples were assessed by independent reviewers (S.A.T. and M.T.d.V.). The degree of CA IX expression was graded by assigning a value of 0 to an absence of staining, a value of 1+ to a weak staining and a value of 3+ to the most positive staining. Slides, which stained with intensity between 1+ and 3+, were classified as moderate (2+). When areas with a different expression degree were present, the intensity of the more extensive area was the one only considered.

The percentage of PCNA-positive cancer cells as compared to the total amount of cancer cells was defined as a labeling index (LI). A LI $< 10\%$ was considered low, and a LI $\geq 10\%$ was considered high.

Histochemistry

Some samples were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.01 M Millonig buffer, pH 7.3, for 3 h, washed in the buffer above, and embedded in the Technovit 7100 resin (Kulzer) according to the manufacturer's instructions. The sites of CA activity were visualized on semithin sections (2 micron thick) from the resin embedded tissue using the cobalt-phosphate precipitation method[19] as detailed.[20] Controls were performed by adding 1×10^{-6} M acetazolamide, a CA inhibitor, to the incubation medium, or by omitting the sodium bicarbonate substrate.

Biochemical Analysis

Several fragments of neoplastic tissue and the apparently normal renal cortex at a distance from the tumour were collected to perform biochemical analyses. L-Glutathione (GSH), as well as oxided L-Glutathione (GSSG) were measured as previously described.[17] The ratio GSH/GSSG was considered as an indicator of the redox state of the tumor. An increased oxidative state of the cell is frequently associated with a decrease in GSH/GSSG ratio, mainly due to increased GSSG levels. Thus considering the GSH/GSSG ratio as an important index of the overall intracellular redox state[17] we categorized our RCCs in two distinct groups: RCCs with GSH/GSSG $< / = 1$ were considered tumors with high oxidative stress, and RCCs with GSH/GSSG > 1 were regarded as tumors with low oxidative stress.

Statistical Analysis

Statistical analysis was performed using a statistical software package (SPSS). Differences in the oxidative stress pattern and prognostic parameters between cases grouped on the basis of immunohistochemical expression of CA IX were analysed using the Chi-square test and Fisher's exact test. The level of statistical significance was set for $p \leq 0.05$.

RESULTS

We evaluated 14 RCCs. Data on clinical, histopathological, immunohistochemical and biochemical analyses are summarized in Table I. The immunohistochemical analysis using anti-CA IX antibody showed in all samples a moderate (+2) or intense (3+) membranous staining signal with only focal, less

TABLE I Cases of RCC investigated in the present study, with their characterization, CA IX expression pattern, and oxidative stress parameters

Case	CAIX	Pattern*	Age	Sex	Grade	Stage	PCNA	Necrosis**	GSH	GSSG	GSH /GSSG***
1	3.00	S	64	M	2.00	3	H	1	–	57	–
2	2.00	S	68	M	2.00	3	H	0	425	146	2.91
3	3.00	M	57	M	1.00	2	H	1	953	326	2.92
4	2.00	S	71	M	2.00	2	H	1	71	203	0.35
5	3.00	T	63	M	2.00	2	M	1	69	506	0.14
6	2.00	M	48	F	1.00	2	M	0	236	1581	0.15
7	3.00	T	41	M	2.00	2	M	1	421	445	0.95
8	3.00	T	47	M	2.00	2	L	1	914	258	3.54
9	2.00	T	76	M	2.00	3	M	1	704	276	2.55
10	2.00	S	48	M	2.00	2	M	0	2157	258	8.36
11	2.00	S	71	F	2.00	3	M	0	1790	156	11.47
12	2.00	S	68	M	2.00	3	M	1	1428	162	8.81
13	3.00	M	71	M	3.00	2	H	1	1274	201	6.34
14	2.00	S	65	F	2.00	2	M	0	2075	260	7.98

* Morphologic pattern of clear cell RCC: S = solid, T = tubular, M = mixed. ** 0: absence of necrosis; 1: RCC with at least 1% of necrosis. *** GSH and GSSG levels were assessed inside the tumours. When the GSH/GSSG index was < 1, the intratumoral redox state was considered low. Alternatively, when the ratio GSH/GSSG was $> / = 1$, the intratumoral redox state was considered high. Fisher exact test was employed to correlate the presence of necrosis with the degree of CA IX expression in RCCs ($p = 0.03$).

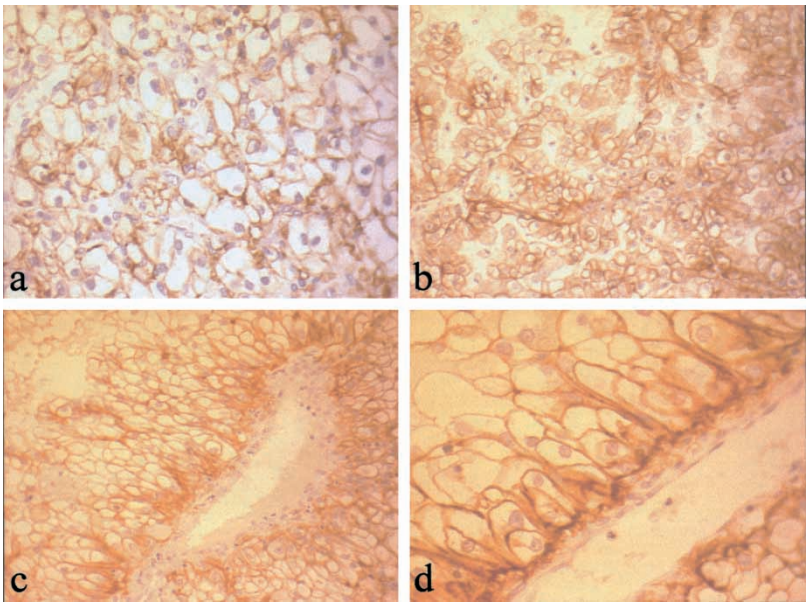

FIGURE 1 (a) Moderate membranous expression of CA IX in solid areas (objective ×25, ocular ×10). (b) More intense staining in tubulo-papillary growth pattern (objective ×10, ocular ×10). (c) Perivascular accentuate expression of CA IX (objective ×10, ocular ×10). (d) Perivascular accentuate expression of CA IX (objective ×40, ocular ×10) (Peroxidase-based detection system with diaminobenzidine—DAB—as chromogen).

extensive areas of weak expression (1+) in some cases. Solid nests (Figure 1a) exhibited a moderate membranous staining in all but 2 cases (3+, cases 1 and 9); The tubular and papillar (Figure 1b) growth pattern showed a more intense expression of CA IX (3+ in 4:5 cases) at the basal side of the cells adjacent to the connective vessels or along the fibrovascular core. In cases 2 and 14 a strong perivascular accentuation of staining was present. The vascular channels showed morphological features of veins of small to medium size (Figures 1c,d).

The histochemical assay documented the presence of carbonic anydrase activity on the tumor cellular membrane most of the time overlapping with CA IX expression. Stratifying cases on the basis of several clinical and istopathological parameters, again no correlation was found for staging, Furhman nuclear grade or PCNA expression. Only the presence of necrosis showed a significant correlation with high CA IX expression. In our small RCCs series, 9 out of 14 RCCs (64.2%) showed at least 1% of necrosis. 6 RCCs (42.9%) exhibited a very high (3+) CA IX expression. The other 9 RCCs (57.1%) showed only a moderate CA IX expression (2+). Notable was that all RCCs with the presence of necrosis displayed the highest degree of CA IX expression (3+). Significant correlation was detected between the presence of necrosis and the degree of CA IX expression ($p = 0.03$, Fisher exact test). Because necrosis may correlate with the local redox state, we further assessed GSH and GSSG levels in tumors. When all the tumors were matched with their respective redox state, no correlation was found with CA IX expression. Stratifying cases for disease-free interval, only the cases alive and well after at least 5 years of follow-up showed an almost significant ($p = 0.057$) relationship between CA IX expression and GSH/GSSG ratio: algebraically lower expression of CA IX was associated with GSH/GSSG > 1 (low oxidative stress), while algebraically higher CA IX expression was associated with GSH/GSSG $< / = 1$ (high oxidative stress). (data not shown)

DISCUSSION

Carbonic anhydrase IX is a transmembrane protein overexpressed in a wide variety of tumor types and induced by hypoxia.[5,9] In this work, we first analyzed by immunohistochemistry the distribution pattern of CA IX in a series of RCC, and then in the same cases we correlated such data with redox state, expressed as GSH/GSSG ratio and the presence of necrosis.

The expression and distribution of CA IX have already been described in RCC. CA IX is a well recognized sensitive and specific marker of clear cell histotype being absent in papillary type, chromophobe and oncocytoma.[21–24] In several solid tumors other than RCC, a close correlation between necrosis and CA IX expression has been reported. CA IX shows a focal perinecrotic pattern in lung, breast, head and neck carcinoma.[25] The importance and the prognostic significance of necrosis in RCC was recognized by Moch.[26] The same data were then confirmed by Cheville in pT1 clear cell carcinoma.[27]

Recently, in a large series of RCC tumor necrosis was found to be significantly correlated with poor prognosis by Sabo.[15]

Because of CA IX widespread expression in RCC it is difficult to investigate its relationship with tumor necrosis. In our small series we showed a further up regulation of CA IX expression in RCCs with at least 1% of necrosis. It has been recently proposed that CA IX may participate in intracellular bicarbonate transport activity.[4] Pyrimidine synthesis may be achieved by bicarbonate via the carbamyl phosphate pathway. Higher CA IX expression in necrotic RCCs might enhance DNA synthesis by raising the intracellular concentration of pyrimidine, increasing tumor cellular growth. In the light of the immunohistochemical results, we confirm as previously seen, the presence of a diffuse and membranous positivity for CA IX exclusively in clear cell RCC histotype. Interestingly, we saw in some cases a perivascular CA IX distribution. The perivascular staining was particularly evident around small veins, small vessels, in the subepithelial connective tissue, in tubular pattern, and along fibrovascular cores in the rare papillary areas of clear cell RCC. Significance of this observation remains unclear.

Again, we correlated the expression of CA IX with some important clinicopathological parameters. Our observations failed to find any significant relationship between the degree of CA IX expression and nuclear grade, tumor stage or the amount of PCNA expression. In RCCs it seems to be controversial. Two different studies found that high-grade and -stage tumors exhibited significantly lower expression[23,24] and in a third study advanced RCC decreased CA IX levels independently associated with poor survival.[25]

Herein for the first time we correlate the expression of CA IX with the intratumoral redox state of RCC. The importance of oxidative stress was underlined by Toyokuni,[16] who proposed a regulatory role in tumor growth and progression. Stratifying the cases for disease free interval, only the patients alive and in good state after at least 5 years of follow-up showed an almost significant relationship between CA IX expression and GSH/GSSG ratio ($p = 0.057$). The lack of statistically significance, is probably due to the very small number of cases. Thus, lower expression of CA IX might be associated with lower oxidative stress, and higher CA IX expression with higher oxidative stress. Further investigations of a consistent number of RCCs, and stratifying the cases for stages and disease free interval, will hopefully clarify the relationship and eventually the role of intracellular redox state on CA IX expression.

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