Carbonic anhydrase IX: a regulator of pH and participant in carcinogenesis

Alessandro Pietro Aldera ^{1,2}, Dhirendra Govender ^{1,3}

¹Division of Anatomical Pathology, University of Cape Town, Cape Town, South Africa
²JDW Pathology Inc, Cape Town, South Africa
³Anatomical Pathology, Pathcare Cape Town, Cape Town, South Africa

Correspondence to

Dr Alessandro Pietro Aldera, JDW Pathology, 19 Upper Camp Road, Maitland 7405, South Africa; aldera@jdwpathology.co.za

Received 30 August 2020

Revised 31 October 2020

Accepted 24 January 2021

Published Online First

22 February 2021

ABSTRACT

Carbonic anhydrase IX (CAIX) is a transmembrane metalloenzyme which is upregulated in tumour cells under hypoxic conditions. CAIX expression is induced by the accumulation of hypoxia-inducible factor-1 α and has several downstream effects, including acidification of the extracellular pH, loss of cellular adhesion and increased tumour cell migration. CAIX is upregulated in a variety of solid organ tumours and has prognostic implications. High CAIX protein expression is a marker of poor prognosis in breast, lung, ovarian and bladder carcinomas. Conversely, low expression is an indicator of poor prognosis in clear cell renal cell carcinoma (CCRCC). CAIX immunohistochemistry is useful diagnostically to identify metastatic CCRCC, and the recently recognised clear cell papillary renal cell carcinoma. There is much interest in targeting CAIX with monoclonal antibodies and small molecule inhibitors. There are several small molecule inhibitors under development which have shown promising results in clinical trials. In this paper, we provide an overview of the role of CAIX in tumourigenesis and outline its use as a prognostic, diagnostic and therapeutic biomarker.

INTRODUCTION

Rapid cell division and growth in solid tumours leads to spatial and flow-based disruption of the microcirculation, and ultimately tissue hypoxia.¹ This leads to activation of a series of adaptive cellular responses, central to which is activation of the transcription factor hypoxia-inducible factor-1 α (HIF-1 α). The accumulation of HIF-1 α causes transcription of downstream effectors, which include vascular endothelial growth factor, erythropoietin, glycolytic enzymes and carbonic anhydrase IX (CAIX).² The carbonic anhydrases are a family of metalloenzymes which are involved with the regulation of intracellular and extracellular pH. Sixteen isoforms have been identified and show different subcellular localisation, catalytic activity and tissue distribution.³

CAIX has been shown to be overexpressed in a variety of solid tumours. Since the identification of CAIX in 1986 (then called G250-antigen), several studies have emerged in the literature exploring its utility as a diagnostic and prognostic biomarker, as well as a potential therapeutic target.⁴ In this paper, we provide an overview of the role of CAIX in the pathogenesis of solid tumours and an update for the practising pathologist of its current role as a diagnostic, prognostic and therapeutic biomarker.

CAIX STRUCTURE, FUNCTION AND NORMAL STATE

Carbonic anhydrases are a family of 16 distinct, highly conserved and ubiquitous zinc metalloenzymes involved in the reversible hydration of carbon dioxide to bicarbonate and a proton. CAIX belongs to the alpha class of carbonic anhydrases, together with CA XII.⁵ CAIX is an integral transmembrane glycoprotein with an extracellular-facing catalytic site and shows the highest hydrogen ion transfer rate among the carbonic anhydrases.⁶ The protein comprises 459 amino acids with the following structure: N-terminal single peptide extracellular region, transmembrane region and intracellular C-terminal region (figure 1). There is a proteoglycan (PG)-like domain adjacent to the extracellular catalytic domain which permits the enzyme to remain active at more acidic pH values.^{7,8} The intracellular tail contains three potential phosphorylation sites, ⁴⁴³T, ⁴⁴⁸S and ⁴⁴⁹Y. Activation of protein kinase A leads to phosphorylation on ⁴⁴³T of CAIX, which results in increased catalytic activity.⁹ Interestingly, dephosphorylation of ⁴⁴⁸S seems to be required for full activation of CAIX.⁹

The chief function of the carbonic anhydrases is the regulation of intracellular pH. The extracellular positioning of the active site allows CAIX to contribute to acidification of the extracellular microenvironment while shuttling bicarbonate into the cytoplasm. The CAIX promoter contains a hypoxia responsive element that binds HIF-1 α , which is the exclusive regulator of its activity.^{2,10}

CAIX expression is limited in normal tissues. In human tissue, expression of CAIX is restricted to the basolateral aspect of proliferating small bowel crypt epithelium,¹¹ efferent ducts in the testis¹² and occasional small foci in pancreatic acini.¹³

ROLE OF CAIX IN TUMOUR PATHOGENESIS

Maintaining a slightly alkaline intracellular pH has been shown to favour tumour cell growth.¹⁴ Inhibition of CAIX results in decreased intracellular pH and negatively influences tumour cell survival.^{15,16} Acidification of the extracellular pH is associated with tumour migration and invasion, extracellular matrix breakdown, chromosomal rearrangements and growth factor production (figure 2).^{15,17} CAIX also appears to contribute to cellular adhesion and migration, both of which are important for metastatic progression in cancer. Svastová *et al*¹⁸ showed in Madin-Darby canine kidney cells that CAIX may modulate E-cadherin-mediated cell adhesion by competing with E-cadherin for binding to β -catenin. CAIX expression has also been linked

© Author(s) (or their employer(s)) 2021. No commercial re-use. See rights and permissions. Published by BMJ.

To cite: Aldera AP, Govender D. *J Clin Pathol* 2021;**74**:350–354.

Figure 1 Carbonic anhydrase IX is a transmembrane glycoprotein which comprises an extracellular catalytic domain with attached PG-like domain, a transmembrane region, and a short intracellular tail containing phosphorylation sites. PG, proteoglycan.

to tumour-associated cell migration by inactivation of rho in a group of cervical carcinoma cells.¹⁹

Tumour suppressors, oncogenes and microenvironmental conditions also appear to play a role in specific circumstances.

Mutation of the tumour suppressor gene *VHL* results in stabilisation of HIF-1 α and promotes hypoxia-independent expression of the HIF-1 α -regulated genes, including CAIX.²⁰ TP53 has also been shown to regulate CAIX expression via HIF-1 α .²¹

Figure 2 Under normoxic conditions, VHL protein binds HIF-1 α , which leads to degradation. Hypoxic conditions cause inactivation of VHL and accumulation of HIF-1 α in the cytoplasm. This causes transcription of CAIX, which is then inserted into the plasma membrane, leading to downstream effects. CAIX, carbonic anhydrase IX; HIF-1 α , hypoxia-inducible factor-1 α ; VHL, von Hippel-Lindau.

Figure 3 Diffuse complete membranous 'box-like' staining with carbonic anhydrase IX is seen in clear cell renal cell carcinoma.

Figure 4 Basolateral 'cup-like' staining with carbonic anhydrase IX immunohistochemistry is diagnostic of clear cell papillary renal cell carcinoma.

ROLE OF CAIX AS A PROGNOSTIC BIOMARKER

CAIX is upregulated in a variety of solid tumours, and there is a relationship between CAIX protein expression and prognosis. High protein expression of CAIX detected by immunohistochemistry is a marker of poor prognosis in breast,²² lung,²³ ovarian²⁴ and bladder²⁵ cancers. High stromal CAIX expression in oral squamous cell carcinoma is associated with increased nodal metastases and decreased survival.²⁶ Haapasalo *et al*²⁷ demonstrated that expression of CAIX predicts poor prognosis in astrocytic tumours. Srivastava *et al*²⁸ recently showed high expression of CAIX to be an independent predictor of poor prognosis in a cohort of 179 hepatocellular carcinoma cases. Conversely, low protein expression of CAIX in clear cell renal cell carcinoma (CCRCC) has been shown to be an independent predictor of poor outcome.^{29–30} de Martino *et al*³¹ examined 54 metastatic CCRCCs and found the CAIX single nucleotide polymorphism rs12553173 to have an overall improved median survival and greater likelihood of response to interleukin therapy.

DIAGNOSTIC UTILITY OF CAIX

CAIX is expressed in 94%–97% of CCRCC and is also detectable in sarcomatoid CCRCC.^{29–32} The pattern of expression is membranous (figure 3) and is typically strong and present in the majority of tumour cells.³³ Expression is comparable using both the M75 and NB100-417 antibody clones.³³ Diffuse CAIX expression is much less frequent in papillary renal cell carcinomas and is not observed in chromophobe carcinoma, collecting duct carcinoma and renal oncocyoma.³⁴ In a study of 366 primary renal tumours, Genega *et al*³⁴ showed that high expression (>85% tumour cells positive) of CAIX occurred in 72.1% (131/184) of CCRCC, and only 8% (4/51) of papillary renal cell carcinoma. Therefore, diffuse CAIX expression is a useful diagnostic marker to separate CCRCC from its morphological mimics, as well as to confirm the diagnosis of metastatic CCRCC.

Clear cell papillary renal cell carcinoma (CCPRCC) is a distinctive low-grade renal epithelial neoplasm which has been recognised in the most recent WHO classification of renal tumours. Tumour cells express CAIX in a diffuse membranous pattern with absent staining along the luminal border, so-called cup-like staining (figure 4).^{35–36} This pattern of CAIX staining is not observed in conventional CCRCC.³⁷ Two other markers

of HIF pathway activation—GLUT1 and HIF-1 α —are typically also overexpressed in CCPRCC. These tumours do not harbour mutations of the *VHL* gene or the typical chromosomal aberrations seen in clear cell or papillary RCC.³⁷

Strong membranous expression of CAIX occurs in haemangioblastoma and can aid in the recognition of this tumour but does not distinguish it from metastatic CCRCC.³⁸

In a recent paper, Nakada *et al*³⁹ showed that CAIX mean protein expression intensity was significantly ($p<0.05$) upregulated in ulcerative colitis-associated colorectal carcinoma (1.55/3) compared with sporadic colorectal carcinoma (0.9/3). CAIX may therefore become a potentially useful marker in distinguishing these.

There has been interest in the use of soluble plasma CAIX for the early detection of cancer. Low levels of serum CAIX are found in patients with CCRCC. Zhou *et al*⁴⁰ showed the serum level of CAIX to be significantly higher in patients with CCRCC compared with patients with non-CCRCC renal tumours.³² High-serum CAIX has also been shown to correlate with tumour size, likelihood of recurrence and the presence of metastatic disease in patients with CCRCC.^{40–41} Ilie *et al*²³ found elevated plasma CAIX to be an independent prognostic biomarker in patients with early-stage non-small cell lung carcinoma.

CAIX AS A POTENTIAL THERAPEUTIC TARGET

Several factors make CAIX a desirable potential target for inhibition. CAIX is selectively expressed in the cell membrane of tumour cells and has an extracellular domain, which makes it amenable to targeting by antibodies and small molecule inhibitors. In vitro studies have shown that carbonic anhydrase (CA) inhibitors, such as acetazolamide, may reduce invasiveness in mouse xenograft models of kidney cancer.⁴²

Two monoclonal antibodies, M75 and G250, are the most well established and studied. M75 targets the PG-like domain of CAIX and is widely used for detection of CAIX in human tumour cells.⁴³ cG250 (a chimeric form of G250) has been developed for immunotherapy (girentuximab) and acts by eliciting antibody-dependent cellular cytotoxicity.⁴⁴ Development of this antibody as a stand-alone therapy was discontinued in phase III trials due to lack of efficacy.

Small molecule inhibitors that are in development, which appear most promising are SLC-0111 (4-(4-fluorophenylureid o)-benzenesulfonamide) and E7070, also known as indisulam (N-(3-chloro-7-indolyl)-1,4-benzenedisulfonamide). SLC-0111 recently completed phase I clinical trials in Vancouver, Canada, for the treatment of solid tumours overexpressing CAIX, and is scheduled to enter phase II clinical trials.⁴⁵ E7070/indisulam showed promise in four phase I clinical trials and is currently in phase II trials in the USA and Europe. In addition to binding CAIX, E7070/indisulam also inhibits the cyclin-dependent kinases leading to cell cycle arrest in the G1/S phase.^{46,47} E7070/indisulam specifically inhibits CA isoforms IX and XII.

The major setback in using small molecules to inhibit CAIX is possible toxicities associated with inhibitors due to their lack of isoform selectivity and specificity. Designing and developing small molecules that specifically target CAIX is of great interest in anticancer therapeutics. Despite the promising results from drug discovery thus far, CA inhibitors are yet to be clinically approved for the treatment of cancer.

Take home messages

- Carbonic anhydrase IX (CAIX) is a transmembrane metalloenzyme, which is responsible for maintenance of intracellular pH.
- Hypoxia causes upregulation of CAIX via activation of the hypoxia-inducible factor-1 α pathway.
- CAIX promotes tumour cell survival and growth by creating an alkaline intracellular pH and also facilitating extracellular matrix breakdown and tumour cell migration.
- CAIX immunohistochemistry has diagnostic utility in recognising clear cell renal cell carcinoma (complete membranous staining) and clear cell papillary renal cell carcinoma (cup-shaped staining).
- CAIX has also emerged as a predictive biomarker in cancer of the lung, colon, breast, cervix, bladder, ovary, brain, head and neck and oral cavity.
- There has been interest in CAIX as a potential target for personalised therapies; however, there are currently no clinically approved therapies.

Handling editor Des Richardson.

Acknowledgements The authors acknowledge Dr Hue-Tsi (Tony) Wu for providing the clear cell papillary renal cell carcinoma case for immunohistochemical staining.

Contributors Both authors contributed equally.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; externally peer reviewed.

ORCID iDs

Alessandro Pietro Aldera <http://orcid.org/0000-0002-9615-1692>

Dhirendra Govender <http://orcid.org/0000-0003-1487-8255>

REFERENCES

- 1 Pries AR, Cornelissen AJM, Sloot AA, *et al.* Structural adaptation and heterogeneity of normal and tumor microvascular networks. *PLoS Comput Biol* 2009;5:e1000394.
- 2 Kaluz S, Kaluzová M, Liao S-Y, *et al.* Transcriptional control of the tumor- and hypoxia-marker carbonic anhydrase 9: a one transcription factor (HIF-1) show? *Biochim Biophys Acta* 2009;1795:162–72.
- 3 Supuran CT. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nat Rev Drug Discov* 2008;7:168–81.
- 4 Oosterwijk E, Ruiter DJ, Hoedemaeker PJ, *et al.* Monoclonal antibody G 250 recognizes a determinant present in renal-cell carcinoma and absent from normal kidney. *Int J Cancer* 1986;38:489–94.
- 5 Hewett-Emmett D, Tashian RE. Functional diversity, conservation, and convergence in the evolution of the alpha-, beta-, and gamma-carbonic anhydrase gene families. *Mol Phylogenet Evol* 1996;5:50–77.
- 6 Wingo T, Tu C, Laipis PJ, *et al.* The catalytic properties of human carbonic anhydrase IX. *Biochem Biophys Res Commun* 2001;288:666–9.
- 7 De Simone G, Supuran CT. Carbonic anhydrase IX: biochemical and crystallographic characterization of a novel antitumor target. *Biochim Biophys Acta* 2010;1804:404–9.
- 8 Innocenti A, Pastorekova S, Pastorek J, *et al.* The proteoglycan region of the tumor-associated carbonic anhydrase isoform IX acts as an intrinsic buffer optimizing CO₂ hydration at acidic pH values characteristic of solid tumors. *Bioorg Med Chem Lett* 2009;19:5825–8.
- 9 Ditte P, Dequiedt F, Svastova E, *et al.* Phosphorylation of carbonic anhydrase IX controls its ability to mediate extracellular acidification in hypoxic tumors. *Cancer Res* 2011;71:7558–67.
- 10 Wykoff CC, Beasley NJ, Watson PH, *et al.* Hypoxia-inducible expression of tumor-associated carbonic anhydrases. *Cancer Res* 2000;60:7075–83.
- 11 Saarnio J, Parkkila S, Parkkila AK, *et al.* Immunohistochemistry of carbonic anhydrase isozyme IX (MN/CA IX) in human gut reveals polarized expression in the epithelial cells with the highest proliferative capacity. *J Histochem Cytochem* 1998;46:497–504.
- 12 Karhumaa P, Kaunisto K, Parkkila S, *et al.* Expression of the transmembrane carbonic anhydrases, CA IX and CA XII, in the human male excurrent ducts. *Mol Hum Reprod* 2001;7:611–6.
- 13 Kivelä AJ, Parkkila S, Saarnio J, *et al.* Expression of transmembrane carbonic anhydrase isoenzymes IX and XII in normal human pancreas and pancreatic tumours. *Histochem Cell Biol* 2000;114:197–204.
- 14 Neri D, Supuran CT. Interfering with pH regulation in tumours as a therapeutic strategy. *Nat Rev Drug Discov* 2011;10:767–77.
- 15 Swietach P, Hulikova A, Vaughan-Jones RD, *et al.* New insights into the physiological role of carbonic anhydrase IX in tumour pH regulation. *Oncogene* 2010;29:6509–21.
- 16 Swietach P, Patiar S, Supuran CT, *et al.* The role of carbonic anhydrase 9 in regulating extracellular and intracellular pH in three-dimensional tumor cell growths. *J Biol Chem* 2009;284:20299–310.
- 17 Parks SK, Chiche J, Pouyssegur J. pH control mechanisms of tumor survival and growth. *J Cell Physiol* 2011;226:299–308.
- 18 Svastová E, Žilka N, Zato'ovicová M, *et al.* Carbonic anhydrase IX reduces E-cadherin-mediated adhesion of MDCK cells via interaction with beta-catenin. *Exp Cell Res* 2003;290:332–45.
- 19 Shin H-J, Rho SB, Jung DC, *et al.* Carbonic anhydrase IX (CA9) modulates tumor-associated cell migration and invasion. *J Cell Sci* 2011;124:1077–87.
- 20 Stillebroer AB, Mulders PFA, Boerman OC, *et al.* Carbonic anhydrase IX in renal cell carcinoma: implications for prognosis, diagnosis, and therapy. *Eur Urol* 2010;58:75–83.
- 21 Kaluzová M, Kaluz S, Lerman MI, *et al.* DNA damage is a prerequisite for p53-mediated proteasomal degradation of HIF-1 α in hypoxic cells and downregulation of the hypoxia marker carbonic anhydrase IX. *Mol Cell Biol* 2004;24:5757–66.
- 22 Lou Y, McDonald PC, Oloumi A, *et al.* Targeting tumor hypoxia: suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. *Cancer Res* 2011;71:3364–76.
- 23 Ilie M, Mazure NM, Hofman V, *et al.* High levels of carbonic anhydrase IX in tumour tissue and plasma are biomarkers of poor prognostic in patients with non-small cell lung cancer. *Br J Cancer* 2010;102:1627–35.
- 24 Choschzick M, Oosterwijk E, Müller V, *et al.* Overexpression of carbonic anhydrase IX (CAIX) is an independent unfavorable prognostic marker in endometrioid ovarian cancer. *Virchows Arch* 2011;459:193–200.
- 25 Klatte T, Seligson DB, Rao JY, *et al.* Carbonic anhydrase IX in bladder cancer: a diagnostic, prognostic, and therapeutic molecular marker. *Cancer* 2009;115:1448–58.
- 26 Brockton NT, Klimowicz AC, Bose P, *et al.* High stromal carbonic anhydrase IX expression is associated with nodal metastasis and decreased survival in patients with surgically-treated oral cavity squamous cell carcinoma. *Oral Oncol* 2012;48:615–22.
- 27 Haapasalo JA, Nordfors KM, Hilvo M, *et al.* Expression of carbonic anhydrase IX in astrocytic tumors predicts poor prognosis. *Clin Cancer Res* 2006;12:473–7.
- 28 Srivastava S, Thakkar B, Yeoh KG, *et al.* Expression of proteins associated with hypoxia and Wnt pathway activation is of prognostic significance in hepatocellular carcinoma. *Virchows Arch* 2015;466:541–8.
- 29 Bui MHT, Seligson D, Han K-ryu, *et al.* Carbonic anhydrase IX is an independent predictor of survival in advanced renal clear cell carcinoma: implications for prognosis and therapy. *Clin Cancer Res* 2003;9:802–11.
- 30 Sandlund J, Oosterwijk E, Grankvist K, *et al.* Prognostic impact of carbonic anhydrase IX expression in human renal cell carcinoma. *BJU Int* 2007;100:556–60.
- 31 de Martino M, Klatte T, Seligson DB, *et al.* CA9 gene: single nucleotide polymorphism predicts metastatic renal cell carcinoma prognosis. *J Urol* 2009;182:728–34.
- 32 Závada J, Závadová Z, Zato'ovicová M, *et al.* Soluble form of carbonic anhydrase IX (CA IX) in the serum and urine of renal carcinoma patients. *Br J Cancer* 2003;89:1067–71.

- 33 Al-Ahmadie HA, Alden D, Qin L-X, *et al.* Carbonic anhydrase IX expression in clear cell renal cell carcinoma: an immunohistochemical study comparing 2 antibodies. *Am J Surg Pathol* 2008;32:377–82.
- 34 Genega EM, Ghebremichael M, Najarian R, *et al.* Carbonic anhydrase IX expression in renal neoplasms: correlation with tumor type and grade. *Am J Clin Pathol* 2010;134:873–9.
- 35 Tickoo SK, Reuter VE. Differential diagnosis of renal tumors with papillary architecture. *Adv Anat Pathol* 2011;18:120–31.
- 36 Srigley JR, Delahunt B, Eble JN, *et al.* The International Society of urological pathology (ISUP) Vancouver classification of renal neoplasia. *Am J Surg Pathol* 2013;37:1469–89.
- 37 Rohan SM, Xiao Y, Liang Y, *et al.* Clear-Cell papillary renal cell carcinoma: molecular and immunohistochemical analysis with emphasis on the von Hippel-Lindau gene and hypoxia-inducible factor pathway-related proteins. *Mod Pathol* 2011;24:1207–20.
- 38 Li M, Song J, Pytel P. Expression of HIF-1 regulated proteins vascular endothelial growth factor, carbonic anhydrase IX and hypoxia inducible gene 2 in hemangioblastomas. *Folia Neuropathol* 2014;52:234–42.
- 39 Nakada N, Mikami T, Horie K, *et al.* Expression of CA2 and CA9 carbonic anhydrases in ulcerative colitis and ulcerative colitis-associated colorectal cancer. *Pathol Int* 2020;70:523–32.
- 40 Zhou GX, Ireland J, Rayman P, *et al.* Quantification of carbonic anhydrase IX expression in serum and tissue of renal cell carcinoma patients using enzyme-linked immunosorbent assay: prognostic and diagnostic potentials. *Urology* 2010;75:257–61.
- 41 Li G, Feng G, Gentil-Perret A, *et al.* Serum carbonic anhydrase 9 level is associated with postoperative recurrence of conventional renal cell cancer. *J Urol* 2008;180:510–4.
- 42 Parkkila S, Rajaniemi H, Parkkila AK, *et al.* Carbonic anhydrase inhibitor suppresses invasion of renal cancer cells in vitro. *Proc Natl Acad Sci U S A* 2000;97:2220–4.
- 43 Zatovicova M, Jelenska L, Hulikova A, *et al.* Carbonic anhydrase IX as an anticancer therapy target: preclinical evaluation of internalizing monoclonal antibody directed to catalytic domain. *Curr Pharm Des* 2010;16:3255–63.
- 44 Surfus JE, Hank JA, Oosterwijk E, *et al.* Anti-renal-cell carcinoma chimeric antibody G250 facilitates antibody-dependent cellular cytotoxicity with in vitro and in vivo interleukin-2-activated effectors. *J Immunother Emphasis Tumor Immunol* 1996;19:184–91.
- 45 Carta F, Vullo D, Osman SM, *et al.* Synthesis and carbonic anhydrase inhibition of a series of SLC-0111 analogs. *Bioorg Med Chem* 2017;25:2569–76.
- 46 Haddad RI, Weinstein LJ, Wiczorek TJ, *et al.* A phase II clinical and pharmacodynamic study of E7070 in patients with metastatic, recurrent, or refractory squamous cell carcinoma of the head and neck: modulation of retinoblastoma protein phosphorylation by a novel chloroindolyl sulfonamide cell cycle inhibitor. *Clin Cancer Res* 2004;10:4680–7.
- 47 Winum J-Y, Poulsen S-A, Supuran CT. Therapeutic applications of glycosidic carbonic anhydrase inhibitors. *Med Res Rev* 2009;29:419–35.