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

Alessandro Pietro Aldera, Dhirendra Govender

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-1α 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. This leads to activation of a series of adaptive cellular responses, central to which is activation of the transcription factor hypoxia-inducible factor-1α (HIF-1α). The accumulation of HIF-1α causes transcription of downstream effectors, which include vascular endothelial growth factor, erythropoietin, glycolytic enzymes and carbonic anhydrase IX (CAIX). 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.

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. 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 CAXII. CAIX is an integral transmembrane glycoprotein with an extracellular-facing catalytic site and shows the highest hydrogen ion transfer rate among the carbonic anhydrases. 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. The intracellular tail contains three potential phosphorylation sites. Activation of protein kinase A leads to phosphorylation on Thr443, Thr448 and Ser449. Activation of protein kinase A leads to phosphorylation on Thr443 of CAIX, which results in increased catalytic activity. Interestingly, dephosphorylation of Ser448 seems to be required for full activation of CAIX.

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 promotor contains a hypoxia responsive element that binds HIF-1α, which is the exclusive regulator of its activity. 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, effrent ducts in the testis and occasional small foci in pancreatic acini.

ROLE OF CAIX IN TUMOUR PATHOGENESIS
Maintaining a slightly alkaline intracellular pH has been shown to favour tumour cell growth. Inhibition of CAIX results in decreased intracellular pH and negatively influences tumour cell survival. Acidification of the extracellular pH is associated with tumour migration and invasion, extracellular matrix breakdown, chromosomal rearrangements and growth factor production. CAIX also appears to contribute to cellular adhesion and migration, both of which are important for metastatic progression in cancer. Studová et al 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...
to tumour-associated cell migration by inactivation of rho in a group of cervical carcinoma cells.\textsuperscript{19}

Tumour suppressors, oncogenes and microenvironmental conditions also appear to play a role in specific circumstances. Mutation of the tumour suppressor gene \textit{VHL} results in stabilisation of HIF-1\textalpha{} and promotes hypoxia-independent expression of the HIF-1\textalpha{}-regulated genes, including CAIX.\textsuperscript{20} TP53 has also been shown to regulate CAIX expression via HIF-1\textalpha{}.\textsuperscript{21}

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.

Figure 2  Under normoxic conditions, VHL protein binds HIF-1\textalpha{}, which leads to degradation. Hypoxic conditions cause inactivation of VHL and accumulation of HIF-1\textalpha{} 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\textalpha{}, hypoxia-inducible factor-1\textalpha{}; VHL, von Hippel-Lindau.
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 membranous (figure 3) and is typically strong and present in the majority of tumour cells.33 Expression is comparable using 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 oncocytoma.36 In a study of 366 primary renal tumours, Genega et al34 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-1α—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 al39 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 al40 showed the serum level of CAIX to be significantly higher in patients with CCRCC compared with patients with non-CCRCC renal tumours.42 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 al23 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.43 44 Development of this antibody as a stand-alone therapy was discontinued in phase III trials due to lack of efficacy.

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 oncocytoma.36 In a study of 366 primary renal tumours, Genega et al34 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.

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.
Small molecule inhibitors that are in development, which appear most promising are SLC-0111 (4-(4-fluorophenylureid o)-benzenesulfonylamide) and E7070, also known as indisulam (N-(3-chloro-7-indoly)−1,4-benzenesulfonylamide). 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. 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. 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-1α 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**

Molecules in pathogenesis


