Carbonic Anhydrase IX: Role in diagnosis, prognosis and cancer therapy
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Front cover photograph: $^{124}$I-cG250 PET/CT (coronal slice of the fused abdominal image) in a patient with multiple left renal masses. Uptake of antibody clearly identifies the ccRCC (solid arrow); the mixed cystic mass (dotted arrow) was not clear cell phenotype. From C. Divgi, page 36.
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Introduction

Carbonic anhydrase IX (CAIX) was first recognized as a potentially important tumour marker in 1986 in a paper published by the Dutch group led by Sven Warnaar. At that time, the identity of the marker was not known and so it was named after the antibody, G250, with which it had been identified. Independently, Zavada, Pastorek and Pastorekova raised the monoclonal antibody M75 against an antigen expressed in mammary tumour MaTu cells and in cervical carcinoma HeLa cells; they coined the term ‘MN’ antigen. Subsequently, working with Stanbridge et al., this antigen was described as a diagnostic marker of cervical carcinoma. It was not long before it became apparent that the G250 and MN antigens were the same; moreover, the antigen was synonymous with an isozyme of carbonic anhydrase that we now call CAIX.

CAIX is a transmembrane enzyme overexpressed in bladder cancer. CAIX is not expressed in normal urothelial tissue, but in 70–90% of TCCs. Expression is usually heterogeneous throughout each tumour and it appears that expression is related to stage and grade. High CAIX expression is associated with increased risk of tumour recurrence and progression, and poor survival. CAIX may be a therapeutic target that can be exploited for both intravesical and systemic treatment.

FIG. 1. Signaling pathways as targets for novel cancer therapies. PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homolog; TSC, tuberous sclerosis complex; mTOR, mammalian target of rapamycin; HIF, hypoxia-inducible factor; VHL, von Hippel–Lindau; Cul, Cullin, ELO, elongins; MAPK, mitogen-activated protein kinase; Hsp90, heat-shock protein 90; VEGF, vascular endothelial growth factor; CKCR4, CXC chemokine receptor-4; EGFR, epidermal growth factor receptor; GLUT, glucose transporter.
Carbonic anhydrase IX: historical and future perspectives

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INTRODUCTION

In 1986 we described monoclonal antibody G[rawitz]250, selected for its remarkable distribution pattern in RCC and normal tissues [1]. There was ample historical evidence that RCC expressed immunological recognizable antigens which was the foundation to search for antibodies which recognized highly RCC-restricted antigens. Immunohistochemical analyses of G250 showed almost homogeneous staining of most RCCs, whereas staining of normal tissues was restricted to a few normal tissues, with most prominent staining in larger bile ducts and gastric mucosa. Remarkably, normal kidney tissue, including foetal kidney tissue did not show any G250 antigen expression. Because G250 expression had already been reported in renal adenomas, we proposed that the induction of G250 expression was possibly due to a common initiating event such as activation of a cellular oncogene product [1]. As we know now, actually the reverse event leads to G250 gene expression, namely inactivation of the von Hippel–Lindau (VHL) tumour suppressor gene product. This explained the ubiquitous expression in most RCCs.

Already in this first description of G250, expression in non-RCC tumours was reported, most prominently in colorectal carcinomas, albeit to a much lower extent. The reason for this non-RCC-associated expression remained unclear. Ongoing research has shown that the heterogeneous expression in non-RCC tumours is hypoxia-related. It was also suggested that monoclonal antibody (mAb) G250 might be useful for RCC scintigraphy in patients. In fact, current efforts suggest that G250 imaging can be used as diagnostic tool, bearing this prediction to reality >20 years after its inception.

In this symposium, we see several different seemingly unrelated lines of research converging, with G250 as common theme. Figure 1 depicts the timeline showing the discovery and development of carbonic anhydrase IX (CAIX), as the molecule is now called. The nomenclature G250/MN/CAIX is a reflection of the different lines of research that have been ongoing in parallel. As described above, G250 indicates the antigen recognized by mAb G250. MN was described in 1992 as part of a two-component system. In 1994 the cloning and characterization of MN was published and in 1996 the gene was shown to be a novel member of the CA family and named CAIX [2]. Subsequent immunohistochemical studies suggested that CAIX was associated with various tumours (e.g. [3,4]), in line with the mAb G250 results. Research on these two seemingly unrelated molecules merged when the mAb G250 target molecule was molecularly characterized and shown to be identical to CAIX/MN [5], hence G250/MN/CAIX.

MONOCLONAL ANTIBODY G250 AND CAIX

Strikingly, the first clinical trials with mAb G250 were already performed and published before the molecular characterization of G250 antigen was achieved. The combined data from the immunohistochemical tissue distribution, animal experiments and ex vivo perfusion of tumour-bearing kidneys that showed high, specific mAb G250 uptake in G250-positive RCC was so persuasive that the human immunology group of Dr L.J. Old at Memorial Sloan-Kettering Cancer Center initiated a phase I protein dose-escalation trial with an in-house produced clinical batch of murine mAb G250. This first (biopsy-based) clinical trial with mAb G250 showed several pivotal aspects: most notably virtually no uptake in other tissues resulting in excellent tumour visualization, and very high tumour uptake [6]. In all candour, the images of the first patients were rather disappointing because there was substantial liver uptake. However, at higher protein doses the liver represented a saturable component and at protein doses of 10 mg only tumour tissue was visualized. Unexpectedly, formally unrecognized disease was visualized, already showing the potential utility of mAb G250 as a diagnostic imaging agent. However, this avenue of research was not pursued mainly because the detection of suspect renal masses and occult metastatic RCC was not deemed advantageous at that time. Additionally, treatment methods for metastasized RCC were poor, and therefore efforts focused on treatment.

The G250 antibody uptake that was seen was up to 10-fold higher than any other mAb uptake in solid tumours, which lead to the design of a phase I/Ii radioimmunotherapy (RIT) trial with murine mAb G250 [7]. RIT lead to stabilization of disease in 17 of 33 patients, with tumour shrinkage in two patients. There was transient liver toxicity, most probably the result of mAb G250 liver uptake, although there was no correlation between the amount of \(^{131}\)I administered or hepatic absorbed radiation dose and the extent and nature of hepatic toxicity. However, antibody immunogenicity restricted therapy to a single infusion, leading to the conclusion that studies with a nonimmunogenic G250 antibody were warranted.

In view of the immunogenicity of murine mAb G250, a chimerized mAb G250 was prepared and in a joint effort the Ludwig Institute for Cancer Research and Centocor produced a clinical batch chimerized mAb G250 for clinical studies. In the protein dose-escalation trial with chimeric G250 (cG250) the primary tumours of all patients with antigen-positive tumours (n = 13), and all known metastases were clearly visualized [8]. Overall uptake, expressed as the percentage of the injected dose (%ID), in the primary tumours was 2.4–9.0. Focally, \(^{131}\)I-cG250 uptake was as high as 0.52 %ID/g. Basically, these results paralleled the findings in the first clinical trial performed with the murine form of mAb G250. However, as expected, cG250 appeared to be immunosilent; minimal human antichimeric antibody (HACA) levels were detected in two of 16 patients [8]. In the subsequent phase I RIT trial, one patient showed a partial response (>9 months) [9], which set the stage for a phase II multidose RIT trial in patients with metastatic RCC. Unfortunately, the clinical results were rather disappointing, with multiple treatment cycles leading to stabilization of disease in a minority of patients [10]. RIT efforts are now focused on more powerful radionuclides such...
as lutetium-177 and yttrium-90 [11]. Indeed, the preliminary results of the 177Lu-G250 trial are quite encouraging, with stabilization and regression of disease in patients with progressive metastatic RCC.

**G250, MN AND CAIX CONVERGE**

The molecular characterization of the antigen recognized by mAb G250 remained problematic. Circumstantial evidence indicated that G250 recognized a bivalent metal ion-dependent conformational epitope, which greatly hampered these efforts. Eventually, screening of an expression library by immunohistochemistry resulted in the isolation of the cDNA encoding for G250 antigen. Sequence analysis showed complete identity with MN/CAIX [5].

The molecular cloning and coming together of the two lines of research also provided insight into the molecular mechanism responsible for the activation of this gene in RCC. After exhaustive analyses of RCC it had become clear that mAb G250 staining was predominantly homogeneous in clear cell (conventional) RCC (ccRCC) [12]. Elegant genetic linkage studies on families with the autosomal dominant inherited von Hippel-Lindau (VHL) syndrome that, amongst others develop ccRCC, had shown that the cause of this syndrome lies in mutations of the VHL gene [13]. Loss of functional VHL protein expression leads to stabilization of specific transcription factors, the so-called hypoxia-inducible transcription factors (HIF). Indeed, loss of functional VHL is causally related to sporadic ccRCC. Studies on CAIX had shown that these factors are an absolute requirement for CAIX expression [14]. Thus, the homogeneous expression of CAIX in ccRCC as seen with mAb G250 could readily be explained by VHL mutations in ccRCC.

The HIF requirement of CAIX expression also explained the seemingly perplexing CAIX expression in various non-RCC tumours: it is a reflection of local hypoxia. Currently CAIX is used as hypoxia marker, more specifically a marker of HIF-1α by many experts in this research field, broadening the interest in this molecule. Although the transcription factors HIF-1α and SP1 have been shown to be indispensable for CAIX transcription [15], further research in this field seems necessary because it is quite possible that CAIX transcription can also occur through combinations of other transcription factors.

**CAIX AND VACCINATION**

In view of the restricted tissue distribution of CAIX and in analogy with vaccine work in melanoma, studies on the possibility to use CAIX as vaccine were initiated using so-called reverse immunology. Indeed, these studies convincingly showed the presence of human leukocyte antigen-restricted T-cell epitopes able to elicit cellular anti-CAIX responses [15–17]. Excitingly, CAIX peptide vaccination resulted in the development of peptide-specific cytotoxic T cells and/or immunoglobulin G reactive to the peptides, showing the potential immunogenicity of CAIX [18]. Patients with multiple lung
metastases showed partial responses, with disappearance and shrinkage of metastatic lesions and stable disease for >6 months. Vaccination of patients with RCC with tumour-RNA pulsed dendritic cells lead to increased anti-CAIX T cell levels, giving credit to the vision that CAIX vaccination can be successful [19]. Recent evidence suggests that strong T cell responses against CAIX exist in patients with acute myeloid leukaemia (AML) [20], broadening the potential applicability of CAIX vaccination beyond RCC. Interestingly, levels of CAIX-reactive T cells was associated with a favourable clinical outcome in patients with AML [20], suggesting that indeed CAIX immunity might play a role in disease control.

CAIX: HYPOXIA MARKER AND MOLECULAR PROGNOSTIC MARKER

Yet another line of research on CAIX was initiated with the realization that CAIX might be a useful surrogate marker for hypoxia. This, combined with the knowledge that hypoxia and poor response to therapy are intimately related, lead to studies investigating the possible prognostic value of CAIX expression. Indeed, high CAIX expression in non-RCC carcinomas is almost invariably linked to a poor prognosis (e.g. [21–26]). This also highlights the suggestion that (sustained) hypoxia may select for an aggressive tumour cell phenotype.

In contrast to non-RCC carcinomas, low CAIX expression (<85%) correlated with poor survival in ccRCC [27,28]. Perhaps the CAIX-negative cells represent a more aggressive tumour component. However, these observations were recently challenged [29].

Interestingly, high (>85%) expression of CAIX in ccRCC has been associated with response to interleukin 2 (IL-2) [30]. Thus, CAIX expression in RCC appears to be linked to various tumour aspects. The correlation between CAIX and prognosis, survival, and response to IL-2 therapy may be the consequence of HIF-directed pathways. Indeed, a strong association has been found between aberrant VHL expression and survival, in line with the CAIX data.

CAIX AS IMMUNOTHERAPEUTIC TARGET

Clearly, passive immunotherapy of patients with RCC has also played a major role in the development of CAIX. The proven tumour-targeting ability and in vitro evidence that mAb G250 treatment could lead to antibody-dependent cellular cytotoxicity [31] spurred several studies aimed at passive immunotherapy. These multi-institutional trials were sponsored by Wilex AG, Munich, Germany, to which mAb G250 was licensed in the late 1990s and Ludwig Institute for Cancer Research. Various (nonrandomised) clinical trials have now been completed with cG250 alone and combined with IL-2 or interferon [32,33]. Thus far, these treatments appear to lead to extended survival time. However, randomised trials are necessary to show this unequivocally in patients with metastatic RCC.

The largest trial, which is currently ongoing, is the adjuvant Adjuvant Rencarex Immunotherapy Phase III Trial to Study Efficacy in nonmetastatic Renal Cell Carcinoma trial. In this phase III randomised, double blind, placebo-controlled trial patients with Eastern Cooperative Oncology Group performance status of 0–with completely resected primary ccRCC and no evidence of remaining local or distant disease, are treated. The study is designed to detect a significant difference between the two treatment arms for disease-free survival; patients will be followed-up long-term to determine overall survival statistics.

CAIX AS DIAGNOSTIC METHOD

As already mentioned in the manuscript describing the first clinical trial, the diagnostic capabilities of cG250 appear outstanding. With a steady increase of incidentally discovered renal masses and new therapeutic methods becoming available, imaging might become important to distinguish more potentially malignant tumours from less aggressive variants. In the first prospective clinical trial with 124I-labelled cG250 a very high specificity and sensitivity to identify ccRCC in patients with suspect renal masses was shown, a clear indication of the potential clinical utility [32,34]. A pivotal registration trial should provide evidence about the value of diagnostic mAb G250 imaging. Whether this imaging method can be used to follow therapy effects remains to be determined.

THE FUTURE OF CAIX

CAIX IN THE TREATMENT OF RCC

In the immediate future, the outcome of the adjuvant G250 trial will provide valuable information on the possibility of adjuvant treatment of high-risk patients. Secondly, the therapeutic lutetium/yttrium trials will reveal the value of these approaches in RCC and will enable direct comparison of two different radionuclides. A positive outcome of these trials will certainly push cG250 toward broader implementation. Secondly, combination trials with cG250 and currently available tyrosine-kinase inhibitors (TKIs) warrant investigation. The working mechanism of these drugs that are approved for metastatic RCC lies mainly in attack of the tumour vasculature, dramatically influencing tumour perfusion. Clearly, in view of perfusion issues, the sequence TKI/mAb G250 needs to be determined, and the possibility to combine these moieties evaluated in preclinical models.

Vaccination studies, also outside of RCC seem of interest in view of the recent results in AML. It may mean that in fact vaccination of all patients with high CAIX expressing tumours might be beneficial. The trial with autologous dendritic cells loaded with adenovirus encoding CAIX-granulocyte/macrophage-colony stimulating factor will be initiated at the University of California-Los Angeles will provide important information about the possibilities along this line of research.

The exquisite tumour accumulation capabilities of mAb G250 almost demand combination with nontargeted moieties. Efforts on antibody-effector molecule fusion proteins such as mAb G250-TNF have shown promise and warrant further study. Combination with functionalized nanoparticles with extraordinary toxic payloads may also lead to alternative methods of treatment. Initial experiments have indeed demonstrated the possibility of this approach.

The development of small molecule drugs specifically aimed at CAIX [35] is certainly of interest. The combination of established CAIX enzymatic activity and tumour specificity might be ideal. Perhaps blockade of CAIX enzymatic activity is detrimental to the tumour environment, leading to significant tumour cell death. One of the challenges is to produce a molecule with high specificity. Obviously, the toxicity profile and nontarget tissue effects will differ dramatically from mAb G250, as the biodistribution profile will differ considerably. Particularly normal
tissues, seemingly less at risk when mAb G250 is used, may be at risk with easy diffusible compounds. Clearly, the results obtained with mAb G250 cannot be transferred to these small molecule drugs.

CAIX IN THE DIAGNOSIS OF RCC AND TO MONITOR THERAPY RESPONSE

The pivotal prospective diagnostic 124I-cG250 imaging trial in patients with suspect renal masses will reveal the value of this approach in RCC. Possibly, cG250 labelled with other positron-emission tomography (PET)-tracers could be superior, but the results with 124I-cG250 have already shown very high specificity and sensitivity. Whether other PET-tracers could improve this significantly remains to be determined. On the other hand, because mAb G250 imaging is directly related to viable tumour tissue, it may be possible to perform TKI therapy-response monitoring with cG250 imaging in addition to MRI that looks at perfusion. In that setting, other PET-tracers may be advantageous. The additional information provided by these images could guide therapeutic decisions. This development may mean that patients will receive multiple injections of mAb G250 during their lifetime. Although the immunogenecity profile of cG250 has been very favourable, there have been HACA responses, particularly in settings where the interval between G250 injections was long. Therefore, humanization of the antibody might be considered.

CAIX AS MOLECULAR PROGNOSTIC MARKER AND PREDICTOR OF THERAPY RESPONSE

Standardization seems necessary in the field of CAIX as a molecular marker for prognosis, survival or therapy response to fully appreciate and define the value of CAIX in these areas. In RCC, the pivotal and most interesting question is what loss of CAIX expression means for RCC cells. Are we observing the development of a more aggressive, CAIX-negative tumour cell population? This does not seem likely in view of the overwhelming number of metastatic RCC lesions that have been visualized in patients with primary ccRCC throughout the years. Nevertheless, the percentage of CAIX-negative cells is closely correlated with prognosis, survival and therapy response in RCC. These two observations are seemingly in contrast. Are ccRCC showing plasticity for CAIX expression? In non-RCC the situation appears to be more straightforward but larger studies under standardized conditions are necessary to establish unequivocally the value of CAIX as a prognostic marker.

ADDITIONAL RESEARCH ISSUES

CAIX AND HYPOXIA

Can we use mAb G250 to visualize hypoxia? Clearly this would be very helpful, as it may allow stratification of patients and make it possible to individualize treatment. However, hypoxia and poor perfusion are intimately related and fluctuating hypoxia may lead to limited CAIX expression. These factors may hamper antibody uptake and accrual. Possibly, CAIX-specific small molecule drugs such as the described sulphonamides may be better suited for this purpose. Rigid, well-controlled animal experiments will provide evidence of whether hypoxia imaging with mAb G250 or sulphonamides is a valid option.

TRANSCRIPTION OF CAIX

Much of CAIX research in non-RCC has focused on the importance of CAIX expression on the regulation of tissue and tumour pH by CAIX, transcriptional aspects, and cell–cell adhesion aspects. Although the primary molecular mechanism involved in CAIX expression has been clearly defined, there are subtle differences when RCC and non-RCC are compared, underscoring that results obtained in RCC cannot be transferred directly to non-RCC. Possibly, transcriptional requirements in RCC differ because HIF interacts with other transcription factors while being fully hydroxylated. Significantly, this situation is also present in the normal tissues that express CAIX such as stomach mucosal cells. Further studies are necessary to deepen our insight and understanding on the activation of CAIX under normoxic conditions.

THE ROLE OF CAIX IN TISSUE pH HOMEOSTASIS

Particularly in non-RCC, the function of CAIX is of great interest, predominantly its role in tissue and intracellular pH regulation. Because pH is tightly controlled, unravelling the CAIX metabolism and composition and the role of the different constituents will certainly improve our basic understanding of pH maintenance. Physiological studies will establish the role of CAIX in normal tissues and, importantly, should also show the transcriptional activation mechanism in the apparent absence of HIF.

CONCLUSION

In conclusion, the versatility of G250/MN/CAIX is extraordinary and it is stunning that one molecule can be used for such different purposes. One molecule as valid therapeutic, diagnostic, and prognostic marker is almost too good to be true. Clearly, much research in these very different research areas is necessary to validate CAIX for these diverse applications. Especially CAIX-guided therapies in ccRCC must be further explored to take advantage of the unique molecular switch that plays such a dominant role in this disease.

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CONFLICT OF INTEREST

EO is a consultant for Wilex AG.

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Abbreviations: UMCN, University Medical Center Nijmegen; VHL, von Hippel-Lindau; mAb, monoclonal antibody; CA, carbonic anhydrase; RIT, radioimmunotherapy; cG250, chimeric G250; %ID, percentage of the injected dose; HACA, human antichimeric antibody; ccRCC, clear cell (conventional) RCC; VHL, von Hippel–Lindau; HIF, hypoxia-inducible factor; AML, acute myeloid leukaemia; IL-2, interleukin 2; TKI, tyrosine-kinase inhibitor; PET, positron-emission tomography.
Molecular mechanisms of carbonic anhydrase IX–mediated pH regulation under hypoxia

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INTRODUCTION

Hypoxia is a crucial factor in tumour physiology that leads to massive molecular and phenotypic changes associated with cancer progression and resistance to treatment. These changes are orchestrated by hypoxia-inducible factor (HIF) and include a shift to oncogenic metabolism, which produces acidosis in the tumour microenvironment. Acidosis has been traditionally attributed to accumulation of lactate and protons excessively produced by glycolysis, extruded from cells and poorly removed by the tumour vasculature. However, experiments with glycolysis-deficient cells indicate that CO₂ is a significant source of acidity in tumours and suggest a role for carbonic anhydrase (CA), a zinc metalloenzyme catalysing the reversible conversion of CO₂ to bicarbonate and proton. There are 15 human CA isoforms that regulate diverse physiological processes based on ion transport and pH balance. CAIX isomerase is induced in different solid tumours in response to hypoxia or inactivating mutation of the von Hippel–Lindau (VHL) tumour suppressor gene, and serves as a hypoxic marker and prognostic indicator. It is an active enzyme with an extracellular catalytic site, and therefore is well positioned to act in control of tumour pH. Recent studies showed that CAIX cooperates with bicarbonate transporters, and participates in extracellular acidification in response to hypoxia. Moreover, its function can be inhibited by CAIX-selective sulphonamides. CAIX can also diminish the intracellular pH gradient in the hypoxic core of three-dimensional tumour spheroids. Reduced CAIX expression in tumour cells perturbs their in vitro survival under hypoxia. Deletion of the catalytic domain from CAIX delays tumour growth in vivo. Interestingly, mutations in intracellular tail of CAIX perturb its pH-regulating function exerted by the extracellular catalytic domain. Altogether, these data show direct functional involvement of CAIX in regulation of pH in tumour microenvironment and suggest novel strategies based on selective CAIX inhibitors for in vivo imaging and therapy of hypoxic tumours.

HYPOXIA AND THE ROLE OF HIF TRANSCRIPTION FACTOR

Solid tumours often develop hypoxic areas due to insufficient supply of oxygen by irregular and functionally defective tumour vasculature. Hypoxia then creates selective pressure in favour of tumour cells that can adapt to this microenvironmental stress. Adaptation to diminished oxygenation has serious consequences: hypoxic tumour cells acquire oncogenic alterations in metabolism, gain increased resistance to conventional anticancer treatment, and are more prone to mutations. Finally, these adaptive changes result in expansion of cells with more aggressive phenotype and increased metastatic potential [1].

Cellular responses to hypoxia include shift to anaerobic glycolysis, angiogenesis, acidosis, reduced cell adhesion, decreased proliferation, and cell death (in severely hypoxic areas). These phenotypic modifications are principally determined at the molecular level by significant alterations of the transcriptional profile of hypoxic cells. The primary molecular response to low oxygen is the stabilization and activation of α subunit of HIF, a key transcriptional regulator of the genes involved in adaptation to hypoxia. In normoxia, HIF-α is modified by oxygen-dependent prolyl hydroxylases (PHDs) and an asparaginyl hydroxylase, factors inhibiting HIF, named FIH, enzymes that all belong to the Fe(II) and 2-oxoglutarate dioxygenase superfamily. FIH hydroxylates asparagine 803 in the C-terminal activation domain of human HIF-1α, thus preventing its interaction with transcriptional coactivators. Prolyl hydroxylases hydroxylate prolines 564 and 402 within the oxygen-dependent degradation domain thus enabling HIF-α recognition by the product of the VHL tumour suppressor gene, followed by its rapid ubiquitylation and proteasome degradation (Fig. 1). Loss or inactivating mutation in VHL, the main negative regulator of the hypoxic pathway, results in development of the hypoxic phenotype also under normoxic conditions in most clear cell RCCs [2,3].

In addition, increased levels and activation of HIF-α may be achieved under normoxic conditions by signal transduction through pathways regulated by activated oncogenes that increase transcription, translation and/or activity of HIF-α and thereby can contribute to or amplify the effects of hypoxia [4–6]. Hypoxia-like responses can be induced in normoxia also by genetic or metabolic events that perturb tricarboxylic acid cycle probably through the accumulation of Krebs cycle intermediates that compete with 2-oxoglutarate to inhibit the HIF hydroxylases [7].

In hypoxia and/or following oncogenic activation or loss of VHL function HIF-α escapes modifications by PHDs and FIH, enters the nucleus, dimerizes with a constitutive β subunit of HIF, interacts with coactivators and forms an active transcriptional complex. This complex binds DNA at sites containing the hypoxia-response element (HRE; 5'-RCGTG-3'), which is present in the promoters of a wide spectrum of target genes. HIF targets include genes encoding the haematopoietic growth factor, erythropoietin, mediators of angiogenesis such as vascular endothelial growth factor (VEGF) and VEGF receptors, enzymes of the glycolytic pathway such as hexokinase 2, lactate dehydrogenase, and glucose transporters (GLUT-1, GLUT-3), and many other genes (Fig. 1). Additional hypoxia-induced genes are involved in regulation of vascular remodelling and plasticity, cell proliferation and viability, cell adhesion, cell matrix metabolism, pH regulation and other cellular processes [8].

HIF-α exists in three basic isoforms (further diversified by alternative splicing). HIF-3α is
the most distantly related isoform, which in one spliced form encodes an inhibitor of HRE-dependent gene expression termed IPAS. On the other hand, HIF-1α and HIF-2α are closely related and mediate transcriptional responses to hypoxia via HRE elements, albeit in apparently distinct temporal, tissue-specific and target-selective patterns [9,10]. This view is supported by phenotypes of knock-out mice and differential roles of HIF-2α vs HIF-1α in the progression of certain tumours. Moreover, many cell types concurrently express both isoforms, but preferentially use one of them to drive expression of certain hypoxia-induced pathways [11,12]. Thus, erythropoiesis and angiogenesis seem to be mediated preferentially by HIF-2α, whereas glycolysis is apparently driven by HIF-1α [13].

ONCOGENIC METABOLISM AND pH REGULATION IN TUMOURS

Diminished oxygen supply to hypoxic tumour areas restricts energy production by oxidative phosphorylation and therefore metabolism of hypoxic cells is shifted to glycolysis, which can generate limited quantities of ATP in absence of oxygen. Underlying mechanism involves HIF-1 coordinated up-regulation of virtually all glycolytic enzymes and glucose transporters via HRE elements in the promoters of their genes [14]. HIF-1 also strongly induces both lactate dehydrogenase isoforms LDH-A and LHD-5, which convert pyruvate to lactate, and pyruvate dehydrogenase kinase 1, which prevents entry of pyruvate into the Krebs cycle, thereby reinforcing the switch to glycolysis over aerobic metabolism [13,15].

Glycolysis is less efficient than oxidative phosphorylation in energy yield; however, its metabolic intermediates can be utilized for biosynthetic reactions leading to production of certain amino acids, nucleotides and lipids. Therefore, glycolysis provides selective advantage to proliferating tumour cells in both anaerobic and aerobic conditions [16]. This provides a rationale for Warburg’s classical finding of high glucose consumption and high lactate production in tumour tissues independently of oxygen availability. Increased expression of the genes coding for glucose transporters and glycolytic enzymes can also be induced in normoxic tumour cells by the AKT pathway and the transcription factor MYC. The AKT serine-threonine kinase enhances glycolytic flux by mobilizing glucose transporters to cell surface as well as by activation of hexokinase 2 that catalyses initial step of glycolysis. The MYC transcription factor can transactivate glycolytic enzyme genes in close cooperation with HIF-1 [17]. Moreover, nonhypoxic oncogenic activation of HIF-1 can also stimulate glycolysis via activation of pyruvate dehydrogenase kinase PDK1 gene reinforcing the phenomenon of increased aerobic glycolysis that Warburg described [18].

Although lactate is the principal end-product of glycolysis, oncogenic metabolism produces also excess of protons and CO₂, which have to be eliminated from tumour cells to maintain neutral intracellular pH that is critical for cell proliferation and survival (Fig. 2). Lactate and protons are extruded by various molecules, including the H⁺/monocarboxylate transporter (MCT), the Na⁺/H⁺ exchanger (NHE), and the vacuolar H⁺/ATP pump, and accumulate in the extracellular space because of insufficient removal via the blood stream consequent on an inadequate tumour vasculature [19–22]. Thereby acid extrusion
results in reduction of extracellular pH (pHe) that is a typical feature of the tumour microenvironment. On the other hand, anion exchangers (AE) and Na+/bicarbonate cotransporters (NBC) import bicarbonate ions that can react with intracellular protons generated by glycolysis, thus increasing cellular production of CO₂ [19]. CO₂ diffuses across the plasma membrane to pericellular space and significantly contributes to extracellular acidosis as shown by experiments with lactate-deficient cells that are still capable of forming acidic tumours in vivo [23].

Extracellular acidification develops especially in tumour regions that are hypoxic as indicated on one hand by the correlation between the mean profiles of partial oxygen pressure and intratumoural pH values, and on the other by VHL/HIF pathway-controlled expression of several components of the pH regulating molecular machinery including AE2, NHE1, and MCT4 [24–26].

Acidic pHe has been associated with tumour progression via multiple effects including up-regulation of angiogenic factors and proteases, increased invasion, and impaired immune functions. Also, it can influence the uptake of anticancer drugs and modulate the response of tumour cells to conventional therapy [27].

CAAs

The recently appreciated involvement of CO₂ in generating microenvironmental acidosis in tumours has suggested a role for CAs. CAs are zinc metalloenzymes catalysing the reversible conversion of CO₂ to bicarbonate and proton in a reaction that involves facilitated hydration of CO₂ to H₂CO₃, followed by the spontaneous dissociation of H₂CO₃ to bicarbonate and proton. CAs are expressed in almost all living organisms and participate in diverse physiological processes based on ion transport and pH balance such as respiration, digestion, renal acidification, bone resorption, etc. The human genome encodes 15 CA isoforms that show variable levels of enzyme activity and differ in molecular features, tissue distribution, expression levels, kinetic properties and sensitivity to inhibitors. They also occupy various subcellular compartments (cytoplasm, mitochondrion, plasma membrane or secretory vesicles) and are engaged in various biochemical pathways.

Twelve catalytically active isozymes (CA I–IV, VA, VB, VI, VII, IX, XII–XIV) possess a shared active site that contains three histidine residues involved in the coordination of a zinc ion with the fourth histidine residue functioning as a proton shuttle [28]. Three acatalytic CA isoforms (VIII, X, XI) lack one of the three critical histidines. In addition, two CA-related proteins that principally function as receptor protein tyrosine phosphatases (RPTPβ/γ) have inactive CA domain pocket, which serves as a receptor site for neuronal adhesion molecule contactin.

FIG. 2. The main components of pH regulation in hypoxic tumour cells. HIF-1-mediated up-regulation of glucose transporters and glycolytic enzymes induces a metabolic shift to glycolysis that produces excess of lactate and protons. To prevent intracellular acidification that is incompatible with the cell growth and survival, these acidic products are extruded by the HIF-regulated MCT4 and the NHE1 and accumulate in the extracellular microenvironment due to insufficient removal via an inadequate vasculature. Oncogenic metabolism also produces high levels of CO₂ that diffuses through the plasma membrane and contributes to the extracellular acidosis, which supports tumour cell invasion. Pericellular CO₂ is hydrated to bicarbonate ions and protons in a reaction catalysed by hypoxia-activated CAIX. CAIX-facilitated production of bicarbonate ions is coupled to AE-mediated transport into the cytoplasm where these ions buffer protons, resulting in neutralization of intracellular pH, and further production of CO₂ that leaves the cell by diffusion and may enter a new round of hydration. GLUT, glucose transporter; TCA, tricarboxylic acid.

CAIX: IDENTIFICATION AND MOLECULAR FEATURES

The molecular identity of CAIX was resolved in 1994 by sequence analysis of its cDNA isolated using the monoclonal antibody (mAb) M75 specific for a plasma membrane antigen detected in human carcinoma cell line HeLa and increased at high cell density in culture [30]. Expression of this antigen, originally named ‘MN’, was found in various tumour cell lines and surgical tumour specimens, but not in the corresponding normal tissues, suggesting its potential usefulness as a tumour marker [31]. Primary structures of the MN cDNA and gene disclosed a large CA domain with a well-conserved active site [30,32]. Because it was the ninth mammalian CA identified, the MN protein was renamed CAIX. In an independent line of research, a RCC-associated antigen detected by mAb G250 was then found to be identical with

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MN/CAIX [33,34] (also reviewed by Oosterwijk elsewhere in this issue).

In addition to the CA domain that displays high enzyme activity, CAIX contains a C-terminal transmembrane anchor followed by a short cytoplasmic tail and an N-terminal extension containing a proteoglycan-like (PG) region that is absent from the other CA isozymes and thus represents a unique feature of CAIX [32]. Since M75 mAb binds to the repetitive epitope in the PG region, it allows selective detection of CAIX without cross-reactivity with other CAs [35]. CAIX is expressed in the form of a 153 kDa trimer composed of disulphide-linked 58/54 kDa monomers, which are glycosylated at asparagine 346 [30,31]. The cytoplasmic tail of CAIX contains three putative phosphorylation sites (T443, S448 and Y449). Of note, epidermal growth factor-induced phosphorylation sites (T443, S448 and Y449).Tail of CAIX contains three putative phosphorylation sites (T443, S448 and Y449). Of note, epidermal growth factor-induced phosphorylation of tyrosine 449 has recently been implicated in signal transduction via the Akt pathway [36].

CAIX exhibits distinct expression pattern characterized by limited expression in normal tissues contrasting with its broad distribution in many different tumours. Normal expression of CAIX is seen in the epithelia of gastrointestinal tract, particularly in the glandular gastric mucosa [37]. Different types of human tumour show high levels of ectopic expression of CAIX in significant proportion of specimens. These include carcinomas of the uterine cervix, kidney, oesophagus, lung, breast, colon, brain, vulva (reviewed by Parkkila in this issue). Comparison of the cDNA sequences of CAIX expressed in HeLa cervical carcinoma cell line and in the stomach showed no difference, indicating that mutations are not responsible for the association of CAIX with tumours and suggesting involvement of cancer-related regulatory pathways in the control of its expression [37].

**CAIX REGULATION**

Expression of CAIX is primarily regulated at the level of transcriptional activation. The promoter of the CA9 gene contains an HRE element (localized just in front of the transcription start site at position –3/–10) that binds HIF-1 and induces transcription of the CA9 gene in response to microenvironmental hypoxia and increased cell density [38]. In both conditions, mitogen-activated protein kinase and phosphatidyl inositol 3-kinase pathways mediate signalling to HIF-1 in an SP1-dependent fashion and cooperate in activation of CA9 transcription [39,40].

In most clear cell RCCs, CAIX is frequently expressed at a high level even in normoxia due to functional inactivation of VHL tumour suppressor gene that is unable to negatively regulate HIF-α [41]. In keeping with this, re-expression of the wild type VHL then leads to decreased level of CAIX in RCC cells [42]. Interestingly, CAIX appears to be an exclusive HIF-1α target in RCC and therefore its expression decreases at more advanced tumour stages together with expression of HIF-1α, which is replaced by HIF-2α as the tumour develops [12].

In addition to transcriptional regulation, control of CAIX expression involves alternative splicing of the CA9 transcript [43], which produces less abundant, hypoxia independent mRNA lacking the exons 8/9 and coding for truncated, cytoplasmic/secreted form of CAIX that displays diminished enzyme activity and behaves in a dominant negative fashion.

Finally, the amount of the full-length transmembrane CAIX protein is regulated post-translationally by metalloproteinasemediated shedding of the ectodomain, which can be induced by treatment with modulators of phosphorylation signalling [44].

Hypoxia-regulated expression of CAIX provides an explanation for its broad distribution in different types of solid tumours and further strengthens the idea of the involvement of CAIX in the molecular machinery regulating the pH of tumour cells.

**CAIX ROLE IN pH CONTROL**

From the functional point of view, CAIX behaves as an adhesion molecule, which on the one hand destabilizes E-cadherin mediated cell–cell contacts by competitive interaction with β-catenin, and on the other mediates attachment of cells to solid support [35,45]. Such behaviour is compatible with known progression-associated attributes of malignant tumour cells that need to disconnect from primary tumour mass and then re-attach to secondary sites.

However, CAIX is primarily functioning as an enzyme with the catalytic domain localized at the extracellular face of the plasma membrane, and this position supports its role in pericellular metabolism of CO2. Interestingly, the enzymatic activity of CAIX is insensitive to high lactate concentrations (in contrast to the other CA isozymes) thus allowing CAIX to work efficiently in the hypoxic tumour microenvironment, which is rich in lactate produced by anaerobic glycolysis [46]. On the other hand, CAIX activity is inhibited by bicarbonate suggesting that it can preferentially catalyse the CO2 hydration arm of the reaction producing bicarbonate ions in pericellular tumour regions loaded with CO2 and deprived of bicarbonate. Because bicarbonate ions are unable to diffuse across the plasma membrane, they cannot contribute to the regulation of intracellular pH unless they are actively transported to cell interior by bicarbonate transporters (i.e. AE and NBC mentioned above). These circumstances create the basis for meaningful spatial and functional cooperation between bicarbonate transporter(s) and CAIX on the surface of tumour cells.

The concept of such a cooperative complex, named a 'metabolon', which is composed of bicarbonate transporter communicating with CA, has been proposed by Vince and Reithmeier [47]. Although the issue of direct physical interaction between the metabolon components remains controversial, their functional cooperation has been proven in various cellular/tissue contexts (e.g. in erythrocytes and renal epithelium) and for different combinations of transporters and CA isozymes (AE1–3, NBC and CAII, CAIV, reviewed in [48]). The main advantage of the bicarbonate metabolon relies in locally concentrated production of bicarbonate immediately coupled with its transport thereby resulting in accelerated flux of bicarbonate ions from one side of the plasma membrane to the other one. The improved flux is particularly important for physiological situations that require excessive pH regulation and/or ion movement, and indeed, tumour hypoxia constitutes such a situation.

We have now two important arguments supporting the functional involvement of CAIX in pH regulation in hypoxic cells with ectopic, constitutive expression of CAIX. First, CAIX contributes to the acidification of the extracellular microenvironment of hypoxic
cells [49], and second, CAIX minimizes the intracellular pH gradient in the core of three-dimensional tumour spheroids indicating that it can help to neutralize intracellular pH of hypoxic tumour cells [50]. These pH-modulating effects of CAIX are apparently related to its catalytic activity producing bicarbonate ions imported to cytoplasm where they enter into a dehydration reaction that titrates intracellular protons and helps to maintain neutral intracellular pH. On the other hand, extracellular hydration of CO₂ results in the net production of protons, which remain outside of the cells and contribute to acidosis (Fig. 2). This may have important implications for cancer progression, because maintenance of neutral intracellular pH is vital for cell proliferation and survival, whereas microenvironmental acidosis contributes to aggressive tumour phenotype by promoting invasion and metastasis [51]. In accord with this view, RNA interference-mediated reduction in CAIX decreased the clonogenic survival of hypoxic tumour cells and expression of a dominant-negative variant of CA with deleted CA domain led to delayed tumour growth in vivo [52 and unpublished results].

Reports that CAIX-mediated pH regulation is increased by hypoxia in cellular models with constitutive, hypoxia-independent level of CAIX can be translated to a conclusion that hypoxia activates the catalytic performance of CAIX. On this basis, we propose that in the natural context, hypoxia can lead to increased expression of CAIX, enhanced catalytic activity, assembly of transport metabolon and increased bicarbonate transport. Interestingly, CAIX can co-immunoprecipitate with anion transporters and to some extent increase the bicarbonate transport even under normoxia [53]. Unfortunately, co-immunoprecipitation data as well as direct measurements of bicarbonate transport under hypoxia are missing and therefore it is not possible to evaluate how tight the interaction of CAIX with AE is, and how strongly induced is the CAIX-facilitated performance of the hypoxic metabolon. Nevertheless, based on pH values measured in culture medium with high buffering capacity, we can assume that the increase in bicarbonate transport under hypoxia greatly exceeds the normoxic transport. The underlying mechanism remains to be elucidated, but it might involve either hypoxia-triggered incorporation of an additional metabolon component, or functional cooperation of diverse metabolic pathways (such as bicarbonate transport and lactate export) leading to formation of a larger metabolon complex. Alternatively, post-translational modifications of the cytoplasmic domains of CAIX and/or AE might result in their increased activity. It is also conceivable that all these events can occur simultaneously and that they are mutually interconnected.

### SELECTIVE INHIBITORS AS TOOLS TO DETECT AND STUDY HYPOXIA-ACTIVATED CAIX

The role of CAIX in pH regulation has been confirmed using a CAIX-deletion variant lacking the catalytic domain, which increased the pHe in hypoxia. Similar effects were achieved with selective inhibitors of CAIX enzyme activity [49].

Notably, a fluorescein-conjugated homosulfanilamide inhibitor (FITC-CAI) could bind only to hypoxic cells that expressed CAIX, but neither to hypoxic cells lacking CAIX nor to normoxic cells. This finding evoked the idea that hypoxia induces the catalytic activity of CAIX (it is well established that inhibitors can bind only to active CAs) possibly via modulated CAIX folding that opens the active site and makes it accessible to the inhibitor. Although the mechanism behind the proposed folding alteration is not known, it has been suggested that the highly acidic PG domain could communicate with a highly basic residues around the conserved zinc-binding histidines and serve as a cover closing or opening the entrance to the catalytic site depending on hypoxia. In favour of this assumption, a CAIX variant lacking the PG domain showed increased (albeit not complete) binding of inhibitor even in normoxia [54]. Based on this ability to recognize CAIX in hypoxic and not in normoxic cells, FITC-CAI became an excellent tool for more detailed investigations of molecular determinants of CAIX catalytic activity under hypoxia. Thorough analysis of a whole series of deletion and mutation variants of CAIX using the FITC-CAI binding assay and correlation of this with acidification capacity led to several important findings indicating an intracellular tail as an important part of the CAIX molecule whose integrity is needed for full enzyme activity (unpublished).

It is quite plausible that the intracellular tail mediates an interaction of CAIX with intracellular proteins that might then modulate the function of the extracellular domain. However, further experimentation is needed to better understand this phenomenon.

### TRANSLATION INTO THE CLINIC

CAIX is not only a subject of intense basic research, but also is attracting considerable attention as a clinically useful molecule. Its strong link to cancer and tight regulation by the HIF pathway offers an opportunity to use CAIX as a surrogate marker of hypoxia in solid tumours, as an indicator of VHL mutation in renal cancer, as a prognostic indicator, and also as a target for immunotherapy and pH-modulating therapy [55,56].

The CAIX-specific mAb M75 that works well in immunohistochemistry is suitable for the routine survey of tumour specimens for CAIX expression for prediction of treatment outcome and patient stratification. Numerous studies clearly support the prognostic/predictive value of increased expression of CAIX that indicates a good prognosis in clear cell RCC due to its link with HIF-1α [12] and a poor prognosis in other tumour types [57]. Moreover, a combination of M75 mAb binding to the N-terminal PG domain and the V/10 mAb recognizing the central CA domain allows for detection of extracellular domain of CAIX shed from tumour cells to body fluids and appears promising for screening/monitoring of patients with cancer [44,58].

The CAIX-specific mAb G250 that binds to a non-denatured form of CAIX within the CA domain (as indicated by its competitive binding with the other CA domain specific Mabs, unpublished) has been quite thoroughly investigated as a tool for in vivo imaging as well as for immunotherapy of RCC [59,60] (for more details see the articles published in this issue).

However, recent experimental data from the study of CAIX-mediated pH regulation indicate that CAIX can also serve as a functional target for in vivo imaging and treatment of hypoxic tumours using inhibitors of the enzyme. These compounds, mostly represented by sulphonamides, sulphanilamides, sulphamates and their derivatives, have already been proposed as anticancer drugs based on their antiproliferative effects in tumour cell lines although due to nonspecific binding to CAs, the isoform target of these inhibitors was not
specified [61]. Therefore, current efforts are focused on increasing the selectivity of the inhibitors towards CAIX by modulating the physical and chemical properties of the compounds via attachment of different side chains and other modifications as described elsewhere [62]. Certain alterations can introduce or improve the membrane impermeability, whereas other changes can modify the size or surface topology that fits better into active site cavities of CAIX than into other isoforms. Some types of modifications have increased the efficiency of inhibitors so that they can inhibit a recombinant catalytic domain of CAIX at subnanomolar concentrations and show a reasonable selectivity for cancer-related CAIX compared with the ubiquitous CAII isoform [63].

As mentioned above, some sulphonamide derivatives represented by the fluorescein-conjugated thioareido-homosulphanilamide, bind only to hypoxic cells that express CAIX [49,64]. Moreover, this FITC-CAI accumulates in the central hypoxic areas of tumour spheroids (unpublished) supporting the view that labelled CAIX-selective inhibitors can be potentially used as tools for in vivo imaging of hypoxic tumours. While CAIX-specific mAbs bind to CAIX protein, which is very stable (with a half life of ~40 h) and remains expressed at the cell surface for a relatively long time after reoxygenation [65], CAIX inhibitors are expected to bind only to enzymatically active CAIX expressed on the surface of hypoxic cells and therefore would image only currently hypoxic tumour areas. Thus, the imaging with CAIX inhibitors might provide different imaging/prognostic information than the mAbs and might be useful particularly in non-RCC tumours in which hypoxia plays an important role in driving tumour progression.

CAIX inhibitors can be potentially useful also in cancer therapy when applied alone or in combination with other modulators of pH control, and/or with conventional chemotherapeutic drugs [66]. Via blocking the CAIX-mediated pH regulation, the inhibitors could prevent intracellular neutralization and simultaneously reduce extracellular acidosis and thus could decrease tumour cell survival and invasion. Even more pronounced effects could possibly be achieved by concurrent treatment with inhibitors of bicarbonate import as well as with inhibitors of lactate and proton-extruding pathways [67]. CAIX inhibition-induced changes in pH gradient across the plasma membrane might also modulate therapeutic responses by influencing the uptake of weakly electrolytic anticancer drugs. It has been already shown that reduction of extracellular acidosis can increase the cytotoxic effects of weakly basic drugs, including doxorubicin and that acetazolamide (a general inhibitor of CA activity) reduces in vivo growth of tumour xenografts when given alone and produced additive tumour growth delays when administered in combination with various chemotherapeutic compounds [68]. However, our current knowledge in this area is insufficient and requires further preclinical experimentation.

**CONCLUSION**

Although a role for CAIX in control of tumour pH has been predicted for almost a decade, only recently obtained experimental data has provide clear experimental support for this. CAIX is now perceived as an active cell surface component of the pH-regulating machinery of hypoxic tumour cells with a functional relevance for cancer progression. As a direct transcription target of the HIF/VHL pathway, it strongly responds to hypoxia by elevated expression and increased catalytic activity. These attributes together with the tight link to various types of tumours make CAIX a promising subject for different types of anticancer therapies. Ongoing research is expected to shed more light into the molecular mechanisms underlying the role of CAIX in cancer progression and to facilitate further analysis of diagnostic and therapeutic strategies based on its detection or inactivation.

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**CONFLICT OF INTEREST**

SP and JP are Patent Inventors; PR is a scientific co-founder of Re-Ox Ltd.

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Abbreviations: HIF, hypoxia-inducible factor; CA, carbonic anhydrase; FIH, factor inhibiting HIF; PHD, prolyl hydroxylase; VHL, von Hippel-Lindau; HRE, hypoxia-response element; VEGF, vascular endothelial growth factor; GLUT, glucose transporter; LDH, lactate dehydrogenase; MCT, H+ / monocarboxylate transporter; NHE, Na+/H+ exchanger; pHe, extracellular pH; AE, anion exchangers; NBC, Na+/bicarbonate cotransporters; mAb, monoclonal antibody; PG, proteoglycan-like; FITC–CAI, fluorescein-conjugated CA inhibitor.
Significance of pH regulation and carbonic anhydrases in tumour progression and implications for diagnostic and therapeutic approaches

Seppo Parkkila
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INTRODUCTION

Carbonic anhydrases (CAs) are involved in cellular pH regulation and have been implicated in some pathogenic processes including tumour progression. In most tumours, intracellular pH is 7.0–7.4, similar to normal cells, whereas extracellular pH is typically 6.9–7.0, although values as low as 6.0 have been reported. The extracellular acidity, which is driven by different ion transport proteins, has been functionally linked to the malignant behaviour of cancer cells. Recently, several studies have identified different CA isozymes in tumours, among which CAII, IX and XII have become the most attractive targets for potential cancer therapy and diagnostic applications. Although CAII is down-regulated in most cancers, it is ectopically expressed in the neovessel endothelium of certain tumours. Recently, an anti-CAI autoantibody response induced by dendritic cell therapy was reported to be associated with a more favourable clinical outcome. CAIX is an attractive ‘cancer-associated’ enzyme that has become a major focus in CA research during the past decade. It is highly expressed in malignancies including renal, ovarian, colorectal, lung, and brain cancers. CAIX research has produced promising therapeutic molecules that are in clinical trials, and CAIX-specific inhibitors are also in ‘the pipeline’. CAIV is another plasma membrane-bound enzyme that is overexpressed in cancer. Although studies on CAIV are still in a preliminary phase, it represents another potential target for therapeutic applications in the future.

CAS – THE KEY ENZYMES IN pH REGULATION

The CAs are zinc-containing proteins that catalyse the reversible hydration of CO$_2$: CO$_2$ + H$_2$O $\leftrightarrow$ HCO$_3^-$ + H$. The $\alpha$-CAs are dominant in vertebrates, and they have different activities, subcellular localizations, tissue distributions and functions. They are involved in many biological processes, such as pH homeostasis, ion transport, respiration, gluconeogenesis, ureagenesis, bone resorption, renal function, as well as formation of saliva, cerebrospinal fluid and gastric acid. Thirteen active isozyme have been identified in mammals to date: five cytoplasmic (CAI, CAII, CAIII, CAVII and CAXIII), five membrane-associated (CAIV, CAIX, CAXII, CAXIV and CAXV), two mitochondrial (CAVA and CAVB) and one secreted form (CAVI) [1–4], Fig. 1 [4], Table 1 [3,5–9]. In addition, a nonclassical form, NonQ/pS4nrb, has been described [10]. There are also three CA-related proteins that belong to the CA gene family, although they lack CA catalytic activity [11]. As with their catalytic activities, all isozymes also differ in their affinity for CA inhibitors (Table 1). Some of the developed CA inhibitors are clinically used as therapeutic agents in the prevention or management of many diseases, such as glaucoma and epilepsy [12–14]. During the last decade, it has become evident that CAs and their inhibitors offer interesting opportunities for both developing novel drugs and diagnostic tools and understanding in greater depth the fundamental processes in which the actions of different CA isozymes are involved.

pH REGULATION IN CANCER

The microenvironment in solid tumours is considered different from the corresponding normal tissues. The interaction between the tumour cells and poorly formed tumour vasculature results in high interstitial fluid pressure, hypoxia, and low extracellular pH [15]. Direct measurement of the pH within a solid tumour has confirmed that the pH is relatively acidic compared with normal tissue. $^{31}$P-magnetic resonance spectroscopy has indicated that the acidity within the tumour is a combination of the extracellular and intracellular pH conditions. Cells within the tumour are capable of maintaining a neutral or slightly alkaline cytosolic pH in the face of external acidosis [16]. Due to its high catalytic activity and expression in tumour cells, CAIX may be greatly involved in ion transport and pH control compared with other CAs that are detectable in tumours. Improved glucose transport, in conjunction with the high rate of anaerobic glycolysis in tumours, generates high levels of lactic acid, which is exported from the cells by monocarboxylate transporters to maintain the neutral intracellular pH needed for cell survival and normal cell functions [17]. The lowered pH of extracellular microenvironment, which results from lactate and proton export, then contributes to an aggressive phenotype of tumour cells that facilitate invasion and metastasis via activation of proteolytic and signal transduction pathways [18,19]. The microenvironmental acidosis can also modulate the response of tumours to anticancer therapy [20]. The lactic acid paradigm has been challenged because even tumours that are impaired in their ability to undergo glycolysis and produce lactate are still able to generate acidic environments [21–23]. The measurements of intratumoural physiological parameters indicate significant contribution of extracellular CO$_2$ [23], which can be hydrated to bicarbonate and a proton by CAIX, which is expressed on the surface of hypoxic tumour cells. The discovery of a transport metabolon, including CAIX and anion exchanger (AE) protein, suggests that CAIX can efficiently utilize CO$_2$ to deliver bicarbonate through the plasma membrane by the AE-driven transport. Intracellular bicarbonate can be converted back to CO$_2$ by cytoplasmic CAI in a reaction that scavenges protons and helps neutralize the cytoplasmic pH. CO$_2$ can then diffuse back to the cell exterior and undergo the next round of CAIX-
such as hypoxia. In fact, RNA interference pH and survive physiological stresses inability to properly neutralize intracellular surface of tumour cells may lead to their were true, elimination of CAIX from the contributing to acidification of the tumour extracellular side of the plasma membrane, mediated hydration [17]. These conversions into milk and saliva. The remaining isozymes are into the cytosol. experiments recently confirmed that CAIX is indeed important for the growth and survival of tumour cells under normoxia and hypoxia [25].

**TABLE 1**

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>CA activity</th>
<th>Affinity for acetazolamide</th>
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<tbody>
<tr>
<td>CAI</td>
<td>Moderate</td>
<td>Moderate</td>
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<tr>
<td>CAII</td>
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<tr>
<td>CAIV</td>
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<td>High</td>
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<tr>
<td>CAV</td>
<td>Moderate</td>
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<td>CAVB</td>
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<tr>
<td>CAVI</td>
<td>Moderate</td>
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<td>CAVII</td>
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<td>CAIX</td>
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<tr>
<td>CAXI</td>
<td>Moderate</td>
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</tr>
<tr>
<td>CAXII</td>
<td>Moderate (mouse)</td>
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<tr>
<td>CAXIV</td>
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<td>High</td>
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<tr>
<td>CAXV</td>
<td>Moderate (mouse)</td>
<td>High</td>
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Higher vertebrate catalytically active α-CAs, their relative CO₂ hydration activity and their affinity for a CA inhibitor, acetazolamide [3,5–9]. Unpublished data on CAXV. The results relate to human isozymes unless otherwise specified.

**FIG. 1.** A typical subcellular localization pattern of the enzymatically active CAs. CAIV and CAXV are anchored to the plasma membrane through a glycosyl phosphatidylinositol linkage. CAXV is not expressed in human tissues [4]. CAIX, CAXII and CAXIV are transmembrane enzymes whose catalytic sites are located to the cell exterior. CAVA and CAVB are mitochondrial enzymes, and CAVI is secreted into milk and saliva. The remaining isozymes are expressed in the cytosol.

CAII, although being the most active and widely expressed isozyme in human tissues, originally attracted only slight attention in cancer research. Gramlich et al. [26] showed that colorectal adenomas and adenocarcinomas failed to stain for CAII. Low expression of CAII in colorectal carcinomas has been recently confirmed in a study where CAI and CAXIII were also investigated in parallel tissue sections [27], as well as in a study where the expression of various CA isozymes was analysed using a microarray technique [28].

CAII is abundantly expressed in the pancreatic duct epithelium, where it is involved in the production of bicarbonate-rich pancreatic juice [2]. In fact, CAII has been considered a marker of pancreatic duct cells and has also been studied in the neoplastic ductal epithelium of the exocrine pancreatic tissue [29]. The expression rate of CAII in the ductal cells, which is sustained after malignant transformation, does not correlate with the malignancy of the tumours, suggesting a limited value for CAII reactivity in diagnosing pancreatic adenocarcinoma.

Leppilampi et al. [30] investigated the presence of CA isozymes in malignant haematoepoietic cell lines and malignant blast cells of bone marrow samples. Three of six malignant haematoepoietic cell lines expressed CAII, whereas no expression was detected for CAI, IX or XII. There were positive reactions in 16 of 26 cases (62%) of acute myeloid leukaemia, 11 of 15 of acute lymphoblastic leukaemia, and one of two cases of chronic myelomonocytic leukaemia. The results indicated that CAII expression is not restricted to one cell lineage but may result from a genetic aberration that occurs in both myeloid and lymphatic lineages or in their progenitor cells.

CAII has been found in brain tumours, including astrocytic tumours, oligodendrogliomas, ependymal and choroid plexus tumours and tumours of nerve sheath cell origin [31]. In brain tumours, a significant fraction of CAII reactivity was recently located to capillary endothelium [32]. Interestingly, ectopic CAII expression has been found in the endothelium of neovessels of several other cancers, including melanoma and oesophageal, renal and lung tumours [33]. Cell culture conditions reminiscent of a cancer cell microenvironment induced CAII expression in endothelial cells in vitro. Experimental data has further suggested that elicitation of an anti-CAII autoantibody response by dendritic cell therapy could be associated with a more favourable clinical outcome.

**CAIX IN NORMAL TISSUES AND CANCER**

Among various normal tissues, CAIX is located mainly in the basolateral plasma membranes of the gastrointestinal epithelium [34]. The highest levels of CAIX have been detected in the mucosa of the stomach and gallbladder. The most intense expression in the intestinal epithelium has been found in the duodenum and jejunum and decreases towards the rectum [35]. By contrast to the gastric epithelium, protein expression in the intestinal epithelium is confined to the rapidly proliferating area, the crypts of Lieberkühn. As the cells migrate along the intestinal villus, they differentiate and coincidentally lose their CAIX expression. These data suggest that CAIX may have a role in the proliferation and differentiation of epithelial cells. In addition to the stomach, gallbladder and intestine, CAIX expression has been detected in the human biliary epithelium, pancreatic ducts, male reproductive organs and mesothelium [34,36–39].

Like the other CAs, CAIX is thought to be involved in pH regulation. It is a highly active
enzyme with catalytic activity quite similar to CAII [40–42]. The enzyme activity of CAIX may be of potential significance for tumour progression because it is thought to be active in the acidification of the extracellular microenvironment surrounding cancer cells, thus facilitating tumour growth and invasion [43,44]. Analogous with another membrane-bound isozyme CAIV [45], CAIX is very likely to interact with AEs in tumour cells by neutralizing the intracellular space. Likewise, the protons produced by CAIX may remain outside and increase the acidosis of the tumour microenvironment [17]. In fact, Morgan et al. [46] recently published the first evidence that CAIX physically and functionally interacts with bicarbonate transport proteins AE1, AE2 and AE3.

It has become evident that CAIX is the most predominant CA isozyme in various tumours, including those that arise from the gastrointestinal tract [5,17]. Many colorectal tumours overexpress CAIX [47], and recently, our group further explored different types of colorectal cancer using both a cDNA microarray and immunohistochemistry [28]. The results indicated that CAIX is the only CA isoform that is overexpressed in hereditary nonpolyposis colorectal cancer (HNPCC). The induction of CAIX expression was greater in HNPCC than in sporadic cancers both at the mRNA and protein levels. Previous studies have shown that CAIX and Ki-67 are colocalized at the site of rapid cell proliferation in colorectal cancer, suggesting that CAIX could be used as a biomarker of increased cell proliferation in the colorectal mucosa [47]. Furthermore, its high expression in premalignant lesions has suggested that it might be a useful marker in early diagnosis of colorectal tumours.

Turner et al. [48] suggested that tumour-associated CAIX may play a role in the proliferation and regeneration in oesophageal squamous epithelium, and loss of its expression might be related to cancer progression in Barrett's-associated adenocarcinomas. Saarnio et al. [36] showed that immunostaining for CAIX was mainly localized to the basolateral surface of the epithelial cells in the biliary epithelial tumours, similar to the normal mucosa [34]. The presence of CAIX in neoplastic hepatobiliary cells and its absence in hepatocellular carcinomas suggested that CAIX could be used as a marker for biliary differentiation in hepatobiliary neoplasms.

Ectopic expression is an important hallmark of CAIX. Thus, CAIX is most abundant in tumours that originate from CAIX-negative tissues. Because the normal gastric mucosa contains the highest levels of CAIX among normal tissues, it was not surprising that gastric carcinomas showed relatively low expression [49]. However, a subgroup of gastric cancers retain CAIX expression in cancer cells at the invasion front [50]. In vitro studies have further indicated that re-expression of CAIX is associated with increased cell invasion; supporting the hypothesis that increased CAIX expression may contribute to a more advanced disease and tumour progression in a subset of gastric cancers.

CAIX may also serve as a valuable marker to predict the prognosis of certain cancers. Its expression has predicted poor survival rate in brain [51] and lung [52,53] cancer, for instance. In lung tumours, the presence of CAIX has specifically been linked to the expression of proteins that are involved in angiogenesis, apoptosis inhibition and cell–cell adhesion disruption, which explains the strong association of this enzyme with poor clinical outcome [54].

Maseide et al. [55] have also reported recently that high CAIX expression predicts a poor prognosis for patients with soft tissue sarcoma. Cervical cancer was one of the first cancer types in which CAIX expression was studied in detail [56]. In 2001, Loncaster et al. [57] showed clinical evidence that CAIX expression in cervical cancer correlates with the levels of tumour hypoxia and associates with a poor prognosis of the disease. The authors suggested that the level of CAIX expression may be used to select patients who would benefit most from hypoxia-modification therapies or bio-reductive drugs.

The current literature already includes several publications on CAIX expression in breast tumours. A study by Bartosova et al. [58] indicated that ectopic activation of the CA9 gene may be implicated in breast carcinogenesis and also suggested potential use of CAIX as a breast tumour marker. The main conclusion from several expression studies is that CAIX correlates with a poor prognosis in breast cancer [59–61], even though Span et al. [62] recently showed that CAIX is more predictive than prognostic in this cancer type.

The expression and localization of CAIX has also been examined in head and neck squamous cell carcinoma (HNSCC) [63]. The enzyme was related to the location of tumour microvessels, angiogenesis, necrosis, and tumour stage and was considered a potential target for future therapy in HNSCC. Recent follow-up studies have included CAIX in the panels of possible predictive markers in HNSCC [64,65]. Although combinations of markers have been associated with treatment outcome, their clinical value as predictive factors must still be established [66].

Some human renal cancer cell lines and renal cancers have shown high expression of CA9 mRNA or CAIX protein [67,68]. The enhanced reverse transcriptase (RT)-PCR assay for CA9 may prove useful in the diagnosis and monitoring of renal cancer [67]. Recently, Sandlund et al. [69] assessed CAIX expression in different subtypes of RCC. They found that expression is higher in conventional rather than other renal cell cancer types, and patients with both conventional RCC and low CAIX expression had a less favourable prognosis. According to the extensive data available on CAIX in renal cancer, the enzyme may indeed represent an ideal marker for clear cell RCC as well as a promising therapeutic target for novel oncological applications, including immunotherapy and radioisotopic methods [17,70].

CAIIX IN CANCER

CAIX is broadly similar in overall structure to CAIX (excluding the proteoglycan-like domain of CAIX). It is also described as a hypoxia-inducible protein, although its localization does not clearly correlate with hypoxic areas within tissues as compared with CAIX. Expression of CAIIX has been studied in normal tissues as well as in several types of cancer. It is expressed in the normal kidney and colon [68,71], and its somewhat heterogeneous expression pattern in tumours may detract from its value as a biomarker [17]. By comparing the current data on CAIIX and CAIX, it is evident that CAIX has a greater potential value as a histological marker protein. Nevertheless, CAIX is physiologically an interesting member of the CA family, and its exact roles deserve further investigation.
behaviour of cancer cells, we designed a study to evaluate the presence of the two forms of CAXII in diffuse astrocytomas, a tumour type known for its aggressive and often incurable behaviour [72]. RT-PCR of tumour samples surprisingly showed that CAXII present in diffuse astrocytomas is mainly encoded by a shorter mRNA variant. We further studied the expression of CAXII in astrocytomas using immunohistochemistry and correlated the results with various clinicopathological and molecular factors. In all, 363 of 370 (98%) cases of diffusely infiltrating astrocytomas showed immunoreactions for CAXII. Importantly, CAXII expression correlated with poorer patient prognosis in univariate analysis of the different isozymes and salivary glands. The observed association between cancers and different CA isozymes has already stimulated translational CA research, which will hopefully lead to novel discoveries that provide new hope for patients with cancer.

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CONFLICT OF INTEREST

SP declares no conflict of interest.

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Abbreviations: CA, carbonic anhydrase; AE, anion exchanger; RT, reverse transcriptase; HNPPC, hereditary nonpolyposis colorectal cancer; HNSCC, head and neck squamous cell carcinoma.
Cancer-associated, hypoxia-inducible carbonic anhydrase IX facilitates CO$_2$ diffusion

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INTRODUCTION

Carbonic anhydrase (CA) enzymes are expressed in virtually all tissues [1] and catalyse the reversible hydration of CO$_2$: CO$_2$ + H$_2$O $\rightleftharpoons$ H$^+$ + HCO$_3^-$.

CO$_2$/HCO$_3^-$ (carbonic) buffer is a major pH buffering system in both the intra- and extracellular compartments. Indeed, among the 13 active isozymes detected to date, some CAs catalyse intracellular reactions whereas others have an extracellular catalytic site [2]. The involvement of H$^+$ ions in the CA-catalysed reaction confers an important pH-regulatory role for the enzyme [3]. Intracellular CA can assist in the removal of metabolically generated acid by augmenting H$^+$ ion titration with intracellular HCO$_3^-$ to membrane-permeant CO$_2$. Extracellular CA can accelerate acid-removal by supplying extracellular substrate for HCO$_3^-$ uptake [e.g. Na$^+$-HCO$_3^-$ co-transport] [4] and by sustaining an outward transmembrane [CO$_2$] gradient, through releasing cell-excreted CO$_2$ to HCO$_3^-$.

One example of an extracellular-facing CA isoform is CAIX. The search for a physiological role of CAIX has attracted significant research effort since it has been shown that CA expression is associated with cancer [5] and is induced by hypoxia via hypoxia-inducible factor (HIF-1$\alpha$) [6]. Tumours are capable of sustaining a high metabolic rate even under inadequate blood-perfusion and hypoxia [7]. For this reason, it has been speculated that tissues expressing CAIX may have enhanced ability to remove metabolically derived acid. Such an adaptation would benefit cancer survival. It has been proposed that CAIX increases extracellular acidification, possibly by shifting the site of CO$_2$ hydration from intra- to extracellular, thereby favouring cell-survival and tumour invasion [8]. Alternatively, CAIX may streamline membrane HCO$_3^-$ transport by delivering substrate through a ‘transport metabolon’ [9]. CAIX may also facilitate extracellular CO$_2$ diffusion by allowing for parallel diffusion of HCO$_3^-$ [3], as has been proposed for other extracellular CA isozymes in muscle [10].

In the present work, we show that CAIX facilitates CO$_2$ diffusion in the unstirred extracellular space of spheroids grown from cancer-derived cell-lines. We also show that this physiological role of CAIX cannot be detected in superfused, single cells because of the nature of the experimental protocol. Facilitated CO$_2$ diffusion may assist acid-removal from poorly perfused tissues, such as tumours.

MATERIALS AND METHODS

RT112 (renal bladder carcinoma cell-line) cells were transfected with the cDNA of human CAIX (a gift from Dr J Pastorek, Bratislava, SK) using a published technique [11]. To grow spheroids, cells were seeded into agarose-coated 96-well plates (=20,000 cells/well in 200 µL of culture medium) and cultured for 72 h [12].

To measure intracellular pH (pH$_i$), cells/spheroids were loaded with the acetoxymethyl-ester of carboxy-SNARF-1 [13] in a chamber mounted on an IRBE microscope (Leica, Germany). Cells/spheroids were superfused at 37 °C with 5% CO$_2$, solution (22 mM HCO$_3^-$, 120 mM NaCl, bubbled with 5% CO$_2$/95% air; pH 7.4) or 20% CO$_2$ solution (88 mM HCO$_3^-$, 54 mM NaCl, bubbled with 20% CO$_2$/80% air; pH 7.4). These superfusates also contained 4.5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 11 mM glucose.

Intracellular carboxy-SNARF-1 was excited by the 514 nm Ar laser-line and fluorescence at 580 nm (spheroids, cells) was monitored. For spheroids, pH$_i$ was averaged in the spheroid core (concentric with spheroid and half its radius) and in the periphery (corona of spheroid of a thickness equal to 10% of spheroid radius) [Fig. 2A].

RESULTS

The resting pH$_i$ in single RT112 cells superfused with 5% CO$_2$ solution was 7.3. Raising superfusate CO$_2$ and HCO$_3^-$ four-fold (at constant solution pH) induced CO$_2$ entry into cells and acidification therein by 0.21 ± 0.01 pH units (Fig. 1A). The rate of acidification was quantified as the time taken to acidify by 0.125 pH units. Under control conditions, the mean (SEM) rate of acidification was 7.6 (0.8) s. The solution manoeuvre was repeated, on the same cells, in the presence of 200 nM 14v [-4-[[5-(aminosulphonyl)-1,3,4-thiadiazol-2-yl]-aminosulphonyl]phenyl]-3,5-nonylene-2,6-dimethyl pyridinium perchlorate, a membrane-impermeant CA inhibitor that selectively blocks extracellular CA isoforms including CAIX [2 min preincubation period was given to allow full drug access] [15]. The mean (SEM) rate of acidification was not significantly different (P > 0.05) in the absence of CAIX activity, at 8.24 (1.0) s.

Similar solution manoeuvres were performed on spheroids (mean (SEM) radius of 299 (22) µm, n = 12), made of RT112 cells (Fig. 2A). Under control conditions, the rate of acidification in the spheroid core was 33% slower than in its periphery (Fig. 2B). Inhibition of CAIX activity with 200 nM 14v did not significantly affect the mean (SEM) rate of acidification in the periphery, at 13.3 (1.0) s in the controls vs 14.6 (1.1) s with 14v (P = 0.063). By contrast, core-acidification was significantly slower in the presence of 14v, at 19.9 (1.9) s in the controls vs 28.1 (4.3) s with 14v (P = 0.024).
The rate of pH change on raising superfusate CO₂ depends on: (i) the supply of extracellular CO₂, (ii) the kinetics of intracellular CA hydration, and (iii) intracellular buffering power.

It is notable that pH changes are much slower in spheroids than in single cells. Intracellular H⁺-buffering capacity is the same in both preparations, as the peak pH change is the same in single cells and spheroids. RT112 cells express low levels of intracellular CA (unpublished results). It is unlikely that the slowing of acidification in spheroids is due to a down-regulation of intracellular CA alone, as this would predict a uniform slowing throughout the spheroid. The difference in acidification rate between spheroids and single cells may be explained in terms of extracellular CO₂ supply. Single cells have an ample supply, delivered continuously in the superfusate, and at equilibrium with HCO₃⁻ and H⁺. Extracellular CAIX could not facilitate a faster CO₂ delivery as, at equilibrium, there would be no net enzyme activity. Inhibiting CAIX with 14v would therefore be expected to have no effect on the CO₂-induced intracellular acidosis, as seen experimentally.

In spheroids, the supply of CO₂ at the surface of constituent cells will be limited by diffusional delays in the unstirred extracellular space. Indeed, as the diffusion distance increases (spheroid periphery to core), acidification slows. Moreover, inhibition of CAIX activity slows intracellular acidification by a greater extent at the spheroid core. These data therefore support a role for CAIX in facilitating CO₂ diffusion across the unstirred extracellular space of multicellular structures. By keeping carbonic buffer nearer to equilibrium, CO₂ levels immediately outside cells can be replenished more rapidly by CAIX-catalysed conversion from HCO₃⁻, which can itself diffuse in parallel with CO₂ from the superfusate. Facilitated CO₂ diffusion in the extracellular space will also result in faster CO₂ removal from respiring cells, particularly at the core of poorly perfused tissues such as developing tumours. It is plausible that the rationale for expressing CAIX in tumours under hypoxic conditions is to assist in acid-removal, thereby protecting the intracellular environment from acidosis that could otherwise impair tumour growth and development.

FIG. 1. Superfused single RT112 cells (n = 30). A, Time-course of intracellular acidification induced by a four-fold increase in [CO₂]. Control vs presence of 14v. B, The rate of acidification is similar in control cells and cells with blocked extracellular CA activity.

FIG. 2. Superfused spheroids (n = 12); pH, measured in the core and periphery. A, Time-course of intracellular acidification induced by a rapid four-fold increase in [CO₂]. Control vs presence of 14v. Inset: cartoon of spheroid, showing peripheral region (blue/green) and core region (red/brown) where pH is measured. During superfusion change, CO₂ diffuses towards the spheroid core. B, The rate of acidification lags by 7 s in the core. This delay is increased two-fold in the presence of 14v.

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CONFLICT OF INTERESTS
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Abbreviations: CA, carbonic anhydrase; HIF, hypoxia-inducible factor; pH, intracellular pH; 14v, [1-[4-[[5-(aminosulphonyl)-1,3,4-thiadiazol-2-yl]aminosulphonyl]phenyl]-3,5-nonylene-2,6-dimethyl pyridinium perchlorate] a membrane-impermeant CA inhibitor.
Carbonic anhydrase IX and renal cell carcinoma: prognosis, response to systemic therapy, and future vaccine strategies

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INTRODUCTION

The discovery of carbonic anhydrase IX (CAIX) occurred around the globe and relied on several independent groups making vital contributions. In 1981, Der and Stanbridge[1] discovered a transmembrane protein in HeLa cell lines that correlated to tumourigenicity and was not found in normal fibroblasts. Pastorek et al.[2] later named this protein ‘MN’ and showed it could induce malignant transformation of fibroblasts. MN was later discovered to be a diagnostic biomarker of cervical cancer[3]. MN was sequenced and its gene product was recognized as an additional member of the CA family and designated CAIX[4]. While Stanbridge and Pastorek were investigating MN, a Dutch group led by Oosterwijk and Warnaar began to identify antibodies that could react to RCC[5,6]. One monoclonal antibody, named G250, was shown to react to 46/47 of RCC primary tumours and seven of eight metastases[5]. Grabmaier et al.[7] later isolated and sequenced the cDNA encoding for G250 and determined that it was homologous to the MN/CAIX gene.

THE FUNCTION OF CAIX

Adequate tissue perfusion is essential for cellular haemostasis. The delicate balance between oxygen supply and demand is disrupted in highly metabolic neoplastic cells. Vascular remodelling by angiogenesis may afford the tumour continued access to oxygen and energy supply. A critical threshold of tissue oxygenation is required for aerobic metabolism and once ATP production halts, cells can no longer maintain cellular gradients. Due to inadequate tissue perfusion and cellular hypoxia, tumour cells frequently rely on high rates of anaerobic metabolism to continue ATP production and survive. Anaerobic reduction of pyruvate generates lactic acid and acidosis can ensue. CAs catalyse the reversible hydration of CO₂ to HCO₃⁻ and H⁺. In RCC it appears that CAIX allows survival in hypoxic and acidic conditions by facilitating transmembrane proton exchange to buffer intracellular pH[8].

CAIX IN OTHER CANCERS

CAIX has limited expression in normal tissues with the exception of gastric mucosa and biliary epithelium[5]. CAIX expression has been reported in a wide variety of nonrenal tumours including cervical, lung, brain, breast, and head and neck cancers (Table 1)[3,9–23]. Expression patterns in these tumours is focal and differs from the diffuse pattern seen in clear cell RCC (ccRCC) with focal CAIX expression distributed in peri-necrotic regions[24]. Higher CAIX expression is associated with poor histological features such as advanced T stage, higher grade, and tumour necrosis in a wide variety of tumours such as astrocytoma and cervical, lung, and breast cancer[3,9–11,25]. High CAIX expression is associated with poor survival in sarcomas and squamous cell carcinomas and may be an independent predictor of poor disease-specific survival (DSS) for astrocytoma, cervical, bladder, and non-small cell lung cancer[10,12–16,25,26]. CAIX expression in breast, lung, and cervical cancer may predict poor response to chemotherapy and external beam radiation[12,15,16].

EXPRESSION IN RENAL TUMOURS

Liao et al.[26] reported the differential expression of CAIX between histological subtypes. All ccRCCs (n = 40) had diffuse expression of CAIX, while papillary and collecting duct subtypes had focal expression. There was no CAIX expression in the two chromophobe RCC specimens[26]. Bui et al.[27] later confirmed the high frequency of diffuse membrane expression for CAIX in ccRCC; in a large cohort of 321 specimens, 94% of tumours expressed CAIX (Fig. 1)[28]. Recently Sandlund et al.[29] reported that five of 14 chromophobe tumours expressed CAIX with a focal expression pattern.

CAIX expression is regulated by the hypoxia-inducible factor 1α (HIF-1α) that accumulates during periods of tumour hypoxia. Mutation of the von Hippel–Lindau (VHL) gene leads to accumulation of HIF-1α and activation of downstream targets including CAIX[24,30]. With up to 60% of sporadic ccRCC tumours having mutations in VHL, and 98% of those showing loss of heterozygocity at the other allele, dysregulation of this pathway leads to diffuse CAIX expression throughout the tumour in the absence of hypoxia[24,31–33]. CAIX expression in non-ccRCC is probably due to the response to hypoxic conditions, as these tumours do not have VHL mutations[32].

CAIX expression in relation to pathological variables

Bui et al.[34] reported CAIX expression had no association with T stage, Fuhrman grade, or lymph node metastasis. There was decreased CAIX expression in patients with worse Eastern Co-Operative Group performance status (ECOG PS). Sandlund et al.[29] recently published similar findings in a large cohort of 228 patients including all histological subtypes. In all RCC subtypes CAIX expression did not correlate with TMN stage, Fuhrman grade, or tumour size. Recently, Patard et al.[35] reported conflicting results showing worse pathological variables with low CAIX expression. An analysis of 100 ccRCC tumour specimens showed decreased CAIX expression was associated with lymph node involvement, higher Fuhrman grade, and larger tumour size. It is unclear why an association with poor prognostic features was found in this cohort. One explanation is that this cohort contained tumours with larger size and higher grades than those analysed by Bui et al.[34].
Identifying high-risk patients remains a clinical challenge. For localized RCC, conventional clinicopathological variables such as TNM stage, ECOG PS, and nuclear grade provide prognostic information but cannot accurately predict disease progression alone. Various prognostic models combine prognostic factors to improve risk group stratification, but many patients have unexpected relapse [36–38]. For metastatic RCC features such as the presence of anaemia, elevated lactate dehydrogenase, multiple sites of metastases, lymph node involvement, sarcomatoid features, and performance status all influence survival [39–41]. Stratifying patients with metastatic disease can identify patients most likely to respond to immunotherapy and who should receive aggressive surgical debulking [42].

The incorporation of molecular markers into conventional models is anticipated to enhance their predictive accuracy. However, molecular models have failed to demonstrate an improvement over existing clinicopathological nomograms in many solid malignancies including prostate cancer [43]. However, in kidney cancer the molecular signature may better predict disease-free survival for localized tumours than clinicopathological data [44]. High-risk patients may benefit from molecular characterization by enrolment into adjuvant trials.

**TABLE 1** CAIX expression on non-renal malignancies

<table>
<thead>
<tr>
<th>Site, reference</th>
<th>Histology</th>
<th>Frequency, %</th>
<th>Associations</th>
<th>Influence on prognosis</th>
<th>Influence on treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical, [3,12]</td>
<td>Squamous</td>
<td>71–94</td>
<td>Higher stage</td>
<td>Independent predictor of DSS and metastasis-free survival</td>
<td>May influence response to radiation therapy</td>
</tr>
<tr>
<td>Bladder, [13,17]</td>
<td>Transitional cell</td>
<td>55 high expression in superficial tumours</td>
<td>None</td>
<td>Possible independent predictor of DFS</td>
<td>Unknown</td>
</tr>
<tr>
<td>Sarcoma, [18,19]</td>
<td>–</td>
<td>66</td>
<td>Unknown</td>
<td>Associated with worse DSS</td>
<td>Unknown</td>
</tr>
<tr>
<td>Brain, [10]</td>
<td>Astrocytoma</td>
<td>78</td>
<td>Higher grade</td>
<td>Independent predictor of overall survival</td>
<td>Unknown</td>
</tr>
<tr>
<td>Breast, [14,15,20]</td>
<td>Ductal</td>
<td>24</td>
<td>Higher grade, necrosis, −ve ER status</td>
<td>May be independent predictor of DSS and DFS</td>
<td>May predict poor response to chemotherapy</td>
</tr>
<tr>
<td>Lung, [9,11,21]</td>
<td>NSCLC</td>
<td>36–80</td>
<td>Necrosis, higher T stage</td>
<td>Independent predictor of DFS</td>
<td>Unknown</td>
</tr>
<tr>
<td>Head and neck, [16,22,23]</td>
<td>Squamous cell</td>
<td>27–90</td>
<td>Higher T stage</td>
<td>Associated with worse overall survival and DFS</td>
<td>May predict poor response to radiation</td>
</tr>
</tbody>
</table>

NSCLC, non-small cell lung cancer; DFS, disease-free survival; ER, oestrogen receptor.

**FIG. 1.** CAIX expression in RCC. Representative tissue core from a normal kidney at ×100 (1) and at ×400 (2), and a representative tissue core of ccRCC with >85% membrane expression at ×100 (3) and at ×400 (4). Reprinted with permission from Leppert et al. [28].

Bui et al. [27] analysed the role of CAIX expression on prognosis in ccRCC. A threshold of 85% CAIX staining allowed risk stratification. Decreased CAIX expression is an independent prognostic indicator of poor survival in patients with metastatic ccRCC (Fig. 2). CAIX status was the greatest predictor of poor outcome with a hazard ratio of 3.1, almost double that of T stage, ECOG PS, or Fuhrman grade. For patients with localized disease, low CAIX expression was associated with worse prognosis, but this failed to reach significance (Fig. 2). Low CAIX expression was useful in identifying a subset of high-risk patients (T classification ≥3 or Fuhrman Grade ≥2) with localized disease. In these patients, median survival was 30 months compared with 10 months for high and low CAIX.
expression, respectively. The sample size was limited \((n = 47)\); however, the differences in survival approached significance \((P = 0.058)\).

We recently reported preliminary results evaluating the prognostic value of CAIX in a prospective study of 32 patients with metastatic ccRCC [44]. There was high CAIX expression in the primary tumour in 62.5% of patients. The 1-year DSS was 83% vs 63% for patients with high and low CAIX expression, respectively. The sample size was 3.9-fold greater \((95\% \ CI 1.2–12.7)\).

CAIX expression might aid selection of patients for IL-2 therapy. Atkins et al. [50] at Harvard later confirmed the association of high CAIX expression with IL-2 response. They analysed 66 patients receiving IL-2, 78% and 51% of responders and nonresponders had high CAIX expression. Prolonged survival of \(>5\) years was only seen in patients with high CAIX expression.

We recently reported preliminary results from a prospective CAIX study at UCLA for patients with metastatic ccRCC. The response rate to IL-2 in patients with high CAIX expression was three of eight, including two complete responders. With multiple therapeutic options now available to oncologists, we think that patients with clinicopathological predictors of response and high CAIX expression should be offered first-line IL-2-based therapy.
amplification of the immune response. Dendritic cells (DCs) are the primary antigen-presenting cells for stimulating T-cell-mediated immune responses. However, DCs account for <10% of leukocytes in renal tumours and activated DCs account for only 0.15% of the total leukocytes [51]. This finding implies that DCs are not effectively recruited and activated by the tumour. The inefficient stimulation of DCs provides a sound rationale for engineering DCs to express tumour-specific antigens, activating them ex vivo, and re-infusing the product as a tumour vaccine.

With high cell surface expression in RCC and limited expression in normal tissue, CAIX serves as an excellent therapeutic target. The UCLA Kidney Cancer Program is currently developing CAIX vaccine strategies. A fusion protein containing two immune activators, CAIX and granulocyte/macrophage-colony stimulating factor (GMCSF) was produced in our laboratory [54]. The fusion protein (GMCSF-CAIX) obtained from a baculovirus expression vector system is a potent immunostimulant with the capacity for activating immunomodulatory DCs and inducing a T-helper cell-supported, CAIX-targeted, and CD8+-mediated antitumour response.

Our current efforts involve the construction of a recombinant adenovirus (Ad) encoding tumour-antigen-fusion gene (Ad-GMCAIX) (Fig. 3). Human immature DCs were generated from CD14+ monocytes derived from peripheral blood mononuclear cells of healthy donors. DCs are infected with Ad-GMCAIX to raise specific cytotoxic T lymphocyte (CTL) responses from autologous peripheral blood lymphocytes (Fig. 4). The expression level of CAIX in the DCs has been confirmed by reverse transcriptase-PCR, flow cytometry, and Western blot. Lymphocytes stimulated by the genetically modified DCs can generate CAIX-specific CTLs. The Ad-GMCAIX significantly increases mature DCs via up-regulation of CD83, CD86 and HLA-DR as compared with unstimulated DCs. Ad-GMCAIX transduced DCs were able to induce CAIX-specific CTLs to lyse RCC cells expressing CAIX in vitro [55]. To make the kidney cancer vaccine available for administration to patients, the UCLA Kidney Cancer Program has collaborated with the National Cancer Institute Rapid Access to Interventional Development programme to bridge the gap between discovery and clinical testing. A phase I/II trial for AdCAIX-GMCSF DC vaccination has been organized to assess its safety and efficacy. The new treatment could be made available to patients with advanced kidney cancer in the summer of 2008.

**CONCLUSIONS**

CAIX is the most important molecular marker in kidney cancer to date. High CAIX expression in other malignancies is associated with hypoxia and appears to be a marker of poor prognosis. However, in ccRCC its presence is linked to VHL mutation and high expression is an independent predictor of improved DSS in metastatic and possibly high-risk localized disease. Additionally, CAIX expression is associated with improved response to IL-2. Patients with good and intermediate clinicopathological features should be considered for immunotherapy especially if they have high CAIX expression. Given the available expression data, we think that at present CAIX is the best and most powerful diagnostic and predictive marker for ccRCC.

**CONFLICT OF INTEREST**

AB is a consultant for Wilex AG and is a chairman for the medical advisory board; the remaining authors declare no conflict of interests.
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Abbreviations: UCLA, University of California-Los Angeles; CA, carbonic anhydrase; DSS, disease-specific survival; ccRCC, clear cell RCC; HIF, hypoxia-inducible factor; VHL, von Hippel-Lindau; EOCG PS, Eastern Cooperative Group performance status; IL-2, interleukin 2; DC, dendritic cell; GMCSF, granulocyte/macrophage-colony stimulating factor; Ad, adenovirus; Ad-GMCAIX, recombinant Ad encoding tumour antigen fusion gene; CTL, cytotoxic T lymphocyte.
Carbonic anhydrase IX as a predictive biomarker of response to kidney cancer therapy

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INTRODUCTION

Renal cancer is the seventh leading malignant condition among men and the twelfth among women. RCC accounts for 85% of renal cancers and about a quarter of patients with RCC have advanced disease at presentation. In addition, a third of patients who undergo surgical resection for localized RCC eventually develop metastasis. Unfortunately, the median survival for patients with metastatic RCC is only ≈13 months [1,2].

There is substantial evidence that RCC is not a single disease but includes different types of malignant epithelial tumours that occur in the kidney [3]. Clear cell RCC (ccRCC) represents the most common form of renal cancer, accounting for 70–80% of cases. Additional RCC types include Type I papillary RCC (5%), Type II papillary RCC (10%), and chromophobe RCC (5%). These different types of renal tumours tend to have distinct clinical courses, and respond differently to treatment. Importantly, different genetic events have been implicated in the development of these malignancies [2,4–6]. ccRCC is the most fatal of all kidney cancer subtypes. In fact, the vast majority of patients with renal cancer that develop metastases have the clear cell histological variant [7].

Radical nephrectomy is the treatment of choice for patients presenting with early stage RCC at the time of diagnosis. Patients with metastatic renal cancer require systemic treatment. While cytotoxic chemotherapy is the treatment of choice for patients with metastatic collecting duct variant renal cancer [8] and has shown some activity in patients with tumours showing sarcomatoid features [9], the activity of chemotherapy in patients with the most common types of metastatic renal cancer is very limited [10]. Cytokine-based immunotherapy with high dose interleukin 2 (IL-2) can produce complete and durable responses in up to 10% of patients with metastatic disease [11,12]. Unfortunately, the treatment is inpatient, toxic and complex; therefore it is frequently limited to selected patients being treated within a few experienced treatment centres.

An improved understanding of the molecular basis of RCC has recently led to the identification of various molecular targets and the development of several therapeutic agents that are active in 50–70% of patients with renal cancer [13]. Indeed, the multikinase inhibitors (MKIs) sorafenib and sunitinib, as well as the mammalian target of rapamycin (mTOR) inhibitor temsirolimus have shown sufficient clinical activity to prompt approval by the USA Food and Drug Administration as single agents in the treatment of patients with advanced kidney cancer [14–16]. Although this represents an important clinical advance in renal cancer, not all the patients treated with these targeted therapies have a substantial clinical benefit and virtually all patients exhibit disease progression at a median of 5–11 months. Moreover, these treatments are also associated with considerable chronic toxicity as well as high costs. Therefore, with multiple partially effective treatments available for patients with metastatic renal cancer, it is important to choose the optimal first-line therapy for individual patients and limit the administration of these various agents to those patients that are likely to benefit. Thus, patient selection strategies for advanced kidney cancer therapies are warranted.

The majority (≈60%) of sporadic ccRCCs are characterized by bi-allelic inactivation of the von Hippel-Lindau (VHL) gene. This genetic event leads to the stabilization of the α-subunit of hypoxia-inducible factor (HIF) and subsequent overexpression of various hypoxia-inducible proteins, including carbonic anhydrase IX (CAIX) [17]. In contrast to many tumours, CAIX overexpression in ccRCC appears to be an early event and is associated with a better prognosis [18]. Recent studies evaluating the relationship of CAIX expression and response to various therapies for advanced kidney cancer suggest that CAIX might be a clinically useful biomarker for selecting patients for certain treatments. In this article, we review the current evidence on the role of CAIX expression as predictor of efficacy for IL-2, temsirolimus and MKIs therapies in RCC.

CAIX AND RESPONSES TO HIGH-DOSE IL-2 THERAPY

High-dose IL-2 therapy remains a valid treatment for advanced kidney cancer but because of its toxicity and high costs efforts to improve patient selection criteria are warranted. In this regard, Upton et al. [19] recently showed that certain histological characteristics of kidney tumours could be utilized to stratify patients into risk groups with significantly different response rates to IL-2 therapy. The study focused on the histopathological evaluation of tumour tissues form 231 patients (163 primary tumours and 68 metastases) who had received IL-2 therapy on Cytokine Working Group (CWG) clinical trials. When primary tumours were analysed, the response rate was higher among patients with ccRCC than in patients with non-ccRCC (21% vs 6%). Importantly, among patients with ccRCC the response rate varied according to histological features of the tumours. In all, 39% of patients with tumours characterized by ‘good’ predictive features [i.e. 50% granular and no granular or papillary features] had responses. By contrast, only 19% of patients with ccRCCs with ‘intermediate’ predictive features [i.e. some alveolar but not papillary features and <50% granular features] and 3% of those with ccRCC containing ‘poor’ predictive features [e.g. >50% granular or any papillary features] had tumour responses. There were similar results when this model was applied to
68 patients with only metastatic tissue specimens available for analysis. Overall, this study suggested that patients with non-ccRCC or ccRCC with ‘poor’ pathological predictive features, respond poorly to IL-2 and should be considered for alternative treatments.

The role of CAIX as a potential predictor of response to IL-2 therapy was first proposed by Bui et al. [18]. By analysing tumour tissue specimens from a cohort of 321 patients with ccRCC, the authors reported that CAIX expression was an independent predictor of poor prognosis in the subpopulation of patients that either developed or presented with metastatic disease. Interestingly, among the 86 patients that had been administered IL-2-based immunotherapy, the overall response rate was 27% in the high-CAIX-staining group as compared with 14% in the low-CAIX-staining group, suggesting a possible association between CAIX expression and IL-2 response.

In an effort to confirm these initial observations, Atkins et al. [20] performed a case-control study on 66 tumours from patients treated with high-dose IL-2 as part of CWG trials. This group of patients was selected to contain a high proportion (41%) of responding patients. CAIX expression was assessed using the M75 antibody and applying the 85% threshold established by the the University of California-Los Angeles (UCLA) group. Pathological features were evaluated according to the Upton criteria. In line with the data from Bui et al. [18] there was a positive association between CAIX levels and IL-2 responses. Indeed, the proportion of tumours with high CAIX levels was significantly higher in the group of responders (78%) than in the nonresponders (51%; odds ratio 3.3, \( P = 0.04 \)). Moreover, patients with high CAIX expression levels in their tumour exhibited significantly longer median survival (\( P = 0.04 \)). Results from CAIX analysis were also subsequently combined with histopathological data. Notably, such analysis led to the development of a two component predictive model in which patients either with ‘good’ pathological predictive features alone or with ‘intermediate’ predictive features and high CAIX expression contained 26 of 27 (96%) responders compared with 18 of 39 (46%) nonresponders. This group of patients had longer median survival and, importantly, contained all the patients that survived for >5 years. Taken together, these results suggest that pathological and molecular tumour features might be eventually used in the clinic to identify optimal patients to receive IL-2 therapy. However, it is important to note that the proposed two-component model was developed in a retrospective fashion in a patient population highly enriched for responders. In an effort to prospectively validate the model and better assess the actual response rates for the ‘good’ and ‘poor’ prognosis groups, the CWG is currently conducting the high-dose IL-2 ‘Select’ trial. Objectives of the trial, which is led by Dr David McDermott at Beth Israel Deaconess Medical Center, also include evaluating whether components of other existing predictive and prognostic models (Memorial Sloan-Kettering Cancer Center, UCLA) can be useful in further defining the patient population that is likely to respond to high-dose IL-2. Finally, the study will also seek to identify other novel biomarkers that might be associated with response to IL-2 enabling potential further refinement of the selection model.

**CAIX AND RESPONSES TO TEMSIROLIMUS**

mTOR is a highly conserved protein kinase that plays a central role in regulating cell growth, proliferation and survival in response to environmental stimuli including growth factors, amino acids, glucose and oxygen availability [21]. Growth factors are known to activate receptor tyrosine kinases that signal through the phosphatidylinositol-3-OH kinase (PI(3)K) pathway and lead to mTOR activation and subsequent stimulation of protein synthesis through the ribosomal protein S6 kinases and the eukaryotic translation initiation factor 4E-binding proteins [22].

Dysregulation of various components of the PI(3)K-mTOR signalling pathway occurs in numerous human cancers and several preclinical models support its direct involvement in tumour development [23]. Recent studies have implicated mTOR in the pathogenesis of RCC supporting its role as therapeutic target for this disease [24,25]. Indeed, there is evidence that mTOR inhibition increases the expression of HIF [26] and that mTOR inhibition exerts antitumour effects in part via down-regulation of HIF activity [27]. In line with these observations, Thomas et al. [28] recently reported that VHL inactivation sensitizes RCC to mTOR inhibition in both a cell culture system and an in vivo xenograft model. Notably, the growth arrest caused by mTOR inhibition correlated with a block in translation of mRNA encoding HIF-1\( \alpha \). Other studies suggest that renal cancers are characterized by activation of the PI(3)K-mTOR pathway and that this feature is associated with a more aggressive tumour behaviour [29].

In accordance with mTOR’s role in kidney cancer, temsirolimus, a specific inhibitor mTOR kinase activity, has shown clinical efficacy in patients with advanced RCC and is now approved for the treatment of this disease. A randomised phase II trial of temsirolimus in RCC showed partial and minor response rates of 7% and 26%, respectively, with a median time to progression of 5.8 months and a median survival of 15.0 months [30]. More recently, in a multicentre phase III trial focused on patients with previously untreated, poor-prognosis metastatic RCC, temsirolimus improved survival when compared with interferon \( \alpha \) [16]. Specifically, patients who received temsirolimus had longer overall survival OS (hazard ratio for death, 0.73; 95% CI 0.58–0.92; \( P = 0.008 \)) and progression-free survival (PFS) (\( P < 0.001 \)) than patients who received interferon alone. Although temsirolimus alone was associated with serious adverse events in fewer patients than was interferon \( \alpha \) alone, toxic effects of temsirolimus included asthenia, rash, anaemia, nausea, dyspnoea, diarrhoea, peripheral oedema, hyperlipidaemia, and hyperglycaemia.

In the attempt to define the subset of patients that are likely to benefit from mTOR inhibition-based therapy, Cho et al. [31] tested the hypothesis that surrogate markers of mTOR pathway activation in pretreatment RCC tissues could predict for response to temsirolimus. To this end, paraffin tissue blocks were obtained from 20 patients that had received this agent as part of the phase II trial and included five patients that had had either partial or minor responses. To study the activation status of the mTOR pathway in tumour tissues, the authors investigated the expression levels of the upstream regulator phosphorylated-Akt (pAkt) and the downstream effector phosphorylated-S6 (pS6). Because of the known effects of mTOR on HIF activity, Cho et al. [31] also interrogated the HIF pathway by studying...
the expression of CAIX and determining the VHL mutational status of the tumours. The results were overall consistent with the initial hypothesis. There was a positive association between higher pS6 expression and response to temsirolimus \( (P = 0.02) \). Accordingly, the median overall survival was greater among patients with high pS6 expression vs those with intermediate or low pS6 expression. Similarly, there was a trend toward a positive association between higher pAkt expression levels and response to temsirolimus \( (P = 0.07) \). In contrast, the response rate appeared similar in patients whose tumours contained either mutant or wild-type VHL. When CAIX expression was analysed using the 85\% threshold previously defined by the UCLA group, there was no association with response. However, none of the patients with very low expression of CAIX (<50\% of positive tumour cells) had objective responses, suggesting a possible association between activation of the HIF pathway and sensitivity to mTOR inhibition. Taken together, results from this analysis seem to imply that patients whose tumours do not display activation of the mTOR pathway (i.e. those with low pS6 and/or pAkt expression) are unlikely to respond to temsirolimus and should be selected for alternative treatments. As these observations were made by a retrospective analysis of a limited number of patients, prospective studies involving a larger number of cases are needed to confirm these findings. Whether markers of HIF pathway activation, and specifically CAIX, can provide additional information on the sensitivity of kidney tumours to mTOR inhibition remains also to be assessed in future investigations. By contrast, an analysis of the pathological variables associated with response to temsirolimus suggested that the benefit of temsirolimus over interferon \( \alpha \) appeared to be most apparent in patients with non-ccRCCs [32], a group of tumours that are unlikely to be associated with VHL mutations. Similar to the IL-2 ‘Select’ trial mentioned above, investigators are planning trials to prospectively validate pS6 as a marker of response to mTOR inhibitors in patients with metastatic RCC as well as to identify other possible predictive biomarkers. That temsirolimus appears to be most beneficial in patients with non-clear cell tumours and in patients with tumours that express pAkt and pS6 suggests that this therapy is most effective in a distinct patient population, that is probably characterized by more aggressive tumours with activation of the PI3K pathway. This finding has encouraged the study of PI3K/AKT pathway inhibition in patients with RCC. It remains to be determined whether a combined treatment regimen involving an mTOR inhibitor and a VEGF pathway blocker will be able to expand the population of sensitive tumours.

**CAIX AND RESPONSES TO MKIS**

MKIs are thought to exert at least part of their antitumour activity in RCC by inhibiting angiogenesis. Indeed, both sunitinib and sorafenib are known to inhibit the receptor tyrosine kinases vascular endothelial growth factor receptor 2 (VEGFR2) and the platelet-derived growth factor receptor \( \beta \) (PDGFR\( \beta \)) expressed in the endothelial cells and pericytes, respectively [24]. As VHL inactivation and subsequent HIF stabilization lead to activation of both the VEGF and PDGFR\( \beta \) pathways, it is more than reasonable to hypothesize that patients whose tumours are characterized by dysregulation of the VHL-HIF pathway are most likely to respond to these therapies.

As part of a project within the Dana-Farber Harvard Cancer Center (DF/HCC) Kidney Cancer SPORE, Signoretti et al. entered into a collaboration with Bayer Pharmaceuticals to assess pathological predictive markers associated with sorafenib therapy. They evaluated slides from 60 patients who had received either sorafenib or placebo as part of the TARGETs trial, a randomised Phase III trial of sorafenib vs placebo in patients whose disease had progressed after cytokine therapy [14]. Three markers, namely CAIX, glucose transporter 1 (GLUT-1) and pS6 were evaluated by immunohistochemistry and specimens were also analysed according to IL-2 pathological risk category. Benefit of sorafenib for tumour shrinkage and PFS relative to placebo appeared stronger in tumours with high CAIX and high GLUT-1 expression. As this preliminary analysis is limited by small sample size and the lack of knowledge regarding the generalisability of this sample set to the overall trial population, investigators are in the process of analysing 200 additional specimens obtained from patients who participated in the TARGETs trial. Additional efforts are underway to evaluate the role of CAIX expression in predicting response to sunitinib.

The preliminary data suggesting that high CAIX and GLUT-1 expression are associated with favourable sorafenib treatment effect (maximum tumour shrinkage and prolonged PFS) are in line with recent findings from Choueiri et al. [33] showing a positive association between the presence of VHL mutations in the patients’ tumours and responses to sorafenib. That both VHL mutations and CAIX levels are informative regarding clinical benefit from sorafenib therapy seems to imply that CAIX expression is a surrogate marker of VHL mutational status in human tumours. To test this hypothesis, Di Napoli et al. [34] recently correlated CAIX expression levels and VHL mutational status in a series of 35 ccRCCs. As expected, VHL mutant tumours expressed significantly higher CAIX levels than wild-type tumours.

As a whole, these recent findings seem to indicate that similar to IL-2, ccRCC tumours with activation of the VHL-HIF pathway are most likely to benefit from VEGFR inhibitor therapy. While this result enhances the difficulty in choosing initial therapy for such patients, it does suggest that combined VEGF-directed therapy and immunotherapy might produce additive effects on this patient population [35,36].

**CONCLUSIONS**

CAIX is a promising biomarker for selection of patients with RCC for both IL-2 and MKI therapy. Prospective validation analyses in larger patient cohorts are being conducted to assess its applicability in the clinical setting. Current investigations are also focusing on the discovery of additional biomarkers that might be used in conjunction with CAIX to improve patient selection strategies. Finally, as current predictive models are largely identified by analysis of primary tumour specimens from the kidney, while treatment is directed against metastatic disease, imaging studies that can evaluate CAIX expression in metastatic lesions might enable further refinement of treatment selection criteria.

**CONFLICT OF INTEREST**

SS and MR declare no conflict of interests; MA is on the Advisory Board for Wilex AG and is a Consultant for CAIX.
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Abbreviations: ccRCC, clear cell RCC; IL-2, interleukin 2; MKI, multikinase inhibitor; mTOR, mammalian target of rapamycin; VHL, von Hippel–Lindau; UCLA, University of California-Los Angeles; HIF, hypoxia-inducible factor; CA, carbonic anhydrase; CWG, Cytokine Working Group; PI(3)K, phosphatidylinositol-3-OH kinase; PFS, progression-free survival; pAkt, phosphorylated-Akt; pS6, phosphorylated-S6; VEGFR2, vascular endothelial growth factor receptor 2; PDGFRβ, platelet-derived growth factor receptor β; GLUT-1, glucose transporter 1.
The use of positron–emission tomography in the diagnosis of tumour phenotype

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INTRODUCTION

Positron-emission tomography (PET) has revolutionized our ability to evaluate metabolic characteristics of cancer. Most studies have been carried out with 18F-fluorodeoxyglucose (FDG), an excellent surrogate for glucose utilization, increased in most cancers. Renal excretion of FDG limits its utility in the detection of primary tumours. However, it is an excellent agent for staging and for detecting metastases.

Several other metabolic tracers are currently being explored as imaging agents in cancer. The lack of promising therapy had limited interest in the development of imaging for metastatic renal cancer. This is changing with the arrival of several biological therapies in this disease. Another factor has been the reduction in the median size of renal masses detected in patients, accompanied by an increasing proportion of patients with relatively benign tumour histology.

The development of 124I-labelled chimeric G250 (cG250), an antibody specific for clear cell RCC (ccRCC), led to speculation that antibody PET may help in noninvasive identification of this aggressive tumour phenotype. This was confirmed in a pilot study with 124I-cG250. A USA Food and Drug Administration (FDA)-approved multicentre trial to assess the accuracy of this novel method is on-going. 124I-cG250 PET may also be useful in early identification of therapeutic response.

G250 recognizes carbonic anhydrase IX (CAIX). Tumour CAIX expression has been shown to be an adverse prognostic factor in many epithelial cancers. Imaging of CAIX antigen expression by PET may therefore be invaluable in the identification of poor-risk patients. Antibody PET with 124I-cG250 is probably the first of many noninvasive techniques to assess unique features of the cancer phenotype.

METABOLIC IMAGING WITH PET

The archetypal PET imaging agent is FDG. Like glucose, FDG is transported into cells by glucose transporters, and is phosphorylated by hexokinase. Unlike glucose, it is not metabolized further, and thus accumulates inside cells. Tumour cells constitutively utilize glucose (the Warburg effect [1]) and hence show greater FDG uptake than normal cells, appearing as areas of increased FDG accumulation [2]. Figure 1 is an example of a patient with RCC studied with FDG-PET/CT. Increased uptake in metastatic osseous and nodal disease is clearly visualized; the primary tumour is also visualized though its detection may not have been evident without concomitant morphological abnormalities.

FDG-PET is increasingly used to stage various neoplasms, and is being incorporated into response assessment algorithms as well [3,4]. The increasing availability of hybrid PET/CT devices, that allow structural CT assessment along with PET, has further enhanced the utility of this noninvasive method.

However, PET with FDG has not become the standard of care for evaluating renal cancer [5]. It has long been felt that renal excretion of tracer (with consequently increased radioactivity in the renal collecting system) limits its utility, though it is becoming increasingly clear that utility may need to be re-assessed, particularly for staging, given the increasing availability of PET/CT.

A relationship between metabolism and signal transduction has been shown in various cancers, with glucose utilization being correlated with Akt signalling [6]. Further, the Warburg effect was attributed not only to increased glucose utilization for the metabolic needs of the cancer cells but also because of altered oxygen pressure and acidity. Thus, altered metabolic signals may be a result of altered phenotype. As in the case of glucose utilization, however, these changes are not specific, a particular alteration is not uniquely accompanied by a metabolic change.

However, identification of metabolic changes is very important, as shown by the utility of FDG-PET, as well as more novel methods for imaging including proliferation assessment with 18F-fluoro-l-thymidine PET [7] and magnetic spectroscopy of choline metabolism [8].

IMAGING TUMOUR-ASSOCIATED ANTIGENS

Cell-surface antigenic features have been widely exploited in immunohistochemistry to characterize the nature of the cancer phenotype [9]. Standardization of such approaches is increasingly being seen as critical to their rational use in treatment decision-making [10]. However, immunohistochemistry is an in vitro test that can only be carried out on tissue amenable to biopsy; it depicts antigenic distribution in a portion of a single tumour location at a particular time, and may therefore not be representative of the antigenic composition of all tumours in the patient.

Whole-body imaging of antigenic distribution therefore has the potential advantages of representing antigenic distribution throughout the body, and it can also be carried out at various times during the course of the disease, and therefore be able to ‘track’ antigenic change. Such imaging has been carried out using radioactive isotopes labelled to intact antibodies; the process has been termed ‘radioimmunodetection’ [11]. The limitation has thus far been the lack of suitable imaging tools, single-photon γ-camera imaging has inherent limitations of sensitivity and specificity, and the only FDA-approved agent to be continued to be used for evaluating any cancer is an indium-111 labelled murine antibody that recognizes the prostate-specific membrane antigen; imaging with this antibody is indicated for re-staging patients [12].

Antibody PET (‘Immuno-PET’) has the advantages of PET, exquisite sensitivity, as
PET FOR DIAGNOSIS OF TUMOUR PHENOTYPE

Iodine-labelled cG250

As has been detailed elsewhere, cG250 is an antibody that has been studied extensively as a therapeutic agent in ccRCC. In summary [14,15], we showed that the antibody had exquisite specificity for ccRCC, and that targeting to both primary and metastatic tumour was excellent as shown by single-photon imaging with $^{131}$I.

The reliable targeting of radio-iodinated cG250 to ccRCC led us to ask whether the antibody could be used to reliably identify ccRCC. During the years that we had been studying renal cancer, the demographic had changed; an increasing proportion of patients with renal masses are now identified serendipitously, and there has been a concomitant decrease in the fraction of patients with ccRCC [16]. The hypothesis proposed was that antibody PET with $^{124}$I-cG250 would reliably detect ccRCC; we decided on at least 90% accuracy. The trial was submitted to the FDA under an Investigational New Drug application sponsored by the Ludwig Institute for Cancer Research. The demographic at Memorial Sloan-Kettering Cancer Center [17], the sole clinical site, led us to expect that we would need to study 54 patients before being able to assess utility, and an early analysis was planned after 15 PET-positive patients.

Patients with renal masses scheduled for surgical removal were entered into the study. Surgery was scheduled for ~1 week after antibody infusion, and PET/CT was carried out before and on the day of surgery.

Fifteen of 16 patients with ccRCC had positive antibody PET, and all nine patients with a different histology had no evidence of antibody accumulation in their tumours. The positive predictive accuracy was 100% [18], and these encouraging results have led to the development of a multicentre trial, sponsored and conducted by Wilex AG, to carry out an adequately powered study to determine the utility of $^{124}$I-cG250 antibody PET in patients with renal masses. Figure 2 is an example of a patient in whom a ccRCC was positive on PET, while a neighbouring cystic tumour was not.

Potential utility of CAIX imaging

CAIX, the target of antibody cG250, is over-expressed in the vast majority of ccRCCs, presumably as a consequence of von Hippel-Lindau mutations, as detailed elsewhere. The relationship between CAIX expression and hypoxia has also been detailed elsewhere, as has the adverse effect of tumour hypoxia upon response to therapy, and survival. CAIX also appears to have value as an independent marker for prognosis and/or aggressive tumour behaviour [19,20]. The availability of an imaging agent that will reliably detect CAIX expression has significant implications for cancer staging and therapy monitoring.

Will antibody PET with $^{124}$I-cG250 become a reliable, noninvasive tool for the identification of CAIX expression in tumours? The clinical trial of course needs to be carried out. The size of the antibody molecule may preclude its penetration into the tumour. However, as the autoradiography in Fig. 3 shows, there was excellent penetration throughout a ccRCC. Heterogeneity of CAIX expression in tumours is another possible limitation, and clinical trials will help address this issue, especially if representative samples of tumour are assessed in vitro.

Conclusion

We have come a long way in our understanding of cancer phenotype, and recent advances in molecular imaging, notably PET with agents that target tumour-associated cell surface molecules, will potentially increase our understanding...
further, leading to the development of tools for stratifying patients with cancer and thence to appropriate patient management. Antibody PET with 124I-cG250 is a seminal first step in the eventual characterization of tumour phenotype using PET.

CONFLICT OF INTEREST

CD is a consultant and study investigator for Wilex AG.

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Abbreviations: PET, positron-emission tomography; FDG, 18F-fluorodeoxyglucose; CA, carbonic anhydrase; cG250, chimeric G250; ccRCC, clear cell RCC; FDA, USA Food and Drug Administration.
INTRODUCTION

Carbonic anhydrases (CAs), a group of ubiquitously expressed metalloenzymes, are involved in numerous physiological and pathological processes, including tumourigenicity, as at least two isoforms, CAIX and CAIXII, are overexpressed in hypoxic tumours. This article discusses the biological/chemical rationale for the novel uses of inhibitors of tumour-associated CA isozymes as diagnostic tools/therapeutic agents for the management of tumours.

A key feature of many tumours is hypoxia [1]. The inadequate supply of oxygen is primarily a pathophysiological consequence of structurally and functionally disturbed microcirculation and deteriorated 02 diffusion processes, being strongly associated with tumour propagation, malignant progression, and resistance to chemo- and radiotherapy [1–3]. Hypoxia regulates the expression of several genes, including a CA isozyme, CAIX, through the hypoxia-inducible factor 1 (HIF-1) cascade. The expression of CAIX is strongly up-regulated by hypoxia and is down-regulated by the wild-type von Hippel–Lindau (VHL) tumour suppressor protein [1–3].

CAIX expression is strongly increased in many types of tumours, such as gliomas, ependymomas, mesotheliomas, papillary/follicular carcinomas, as well as carcinomas of the bladder, uterine cervix, kidneys, oesophagus, lungs, head and neck, breast, brain, vulva, and squamous/basal cell carcinomas, among others [1–4]. In some cancer cells, the VHL gene is mutated leading to a strong up-regulation of CAIX (up to 150-fold) because of constitutive HIF activation [1–4].

CAIX belongs to the highly active human α-CAs, its catalytic properties for the CO2 hydration reaction being comparable with those of the highly evolved catalyst CAII [1,2]. As for all α-CAs, CAIX is susceptible to inhibition by anions and sulphonamides/sulphamates, with the inhibitors coordinating directly to the zinc ion within the active site cavity and participating in various other favourable interactions with amino acid residues situated both in the hydrophobic and hydrophilic halves of the active site. Many low nanomolar CAIX inhibitors have been identified in the last several years [1,2]. Among them, some sulphamates and sulphonamides were characterized by X-ray crystallography and homology modelling [5,6]. Such studies also evidenced compounds that are membrane impermeable (and thus specifically inhibit CAIX in vivo). Both heterocyclic, aromatic sulphonamides as well as aliphatic sulphonamides/sulphamates/sulphamides possessing low nanomolar inhibitory activity against CAIX have been detected to date.

As described, hypoxia, through the HIF cascade, leads to a strong over-expression of CAIX in many tumours. The overall consequence of this is a pH imbalance, with most hypoxic tumours having acidic pH values of ≈6, in contrast to normal tissue which has characteristic pH values of ≈7.4 [7]. Constitutive expression of human CAIX was recently shown to decrease extracellular pH (pH,) in Madin–Darby canine kidney epithelial cells [7]. CAIX-selective sulphonamide inhibitors (of types 1 and 2, Fig. 1) reduced the medium acidity by inhibiting the catalytic activity of the enzyme, and thus the generation of H+, binding specifically to hypoxic cells expressing CAIX. Deletion of the CA active site was also shown to reduce the medium acidity, but a sulphonamide inhibitor did not bind to the active site of such mutant proteins. Therefore, tumour cells decrease their pH, both by production of lactic acid (due to the high glycolysis rate), and by CO2 hydration catalysed by the tumour-associated CAIX, possessing an extracellular catalytic domain [1,2,7]. Low pH, has been associated with tumorigenic transformation, chromosomal rearrangements, extracellular matrix breakdown, migration and invasion, induction of the expression of cell growth factors and protease activation. CAIX probably also plays a role in providing bicarbonate to be used as a substrate for cell growth, whilst it is established that bicarbonate is required in the synthesis of pyrimidine nucleotides [6].

Indisulam (Fig. 1; structure 3), a sulphonamide derivative (originally called E7070) with powerful anticancer activity, was recently shown to act as a CAIX nanomolar inhibitor [8]. Its detailed mechanism of action is not clear, but it is known to be involved in the perturbation of the cell cycle in the G1 and/or G2 phases, the down-regulation of cyclins, the reduction of cyclin-dependent kinase 2 activity, the inhibition of retinoblastoma protein phosphorylation and differential expression of molecules known to participate in cell adhesion, signalling and immune response, in addition to its CAIX inhibitory properties. Indisulam showed in vivo efficacy against human tumour xenografts in nude mice, exhibiting a significant antitumour effect and progressing to Phase I and II clinical trials for the treatment of solid tumours [8].

Among the many derivatives reported to date, some of the most interesting potent CAIX inhibitors are the compounds investigated by Svastova et al. [7] (Fig. 1; structures 1 and 2). Derivative 1 is a fluorescent sulphonamide that binds only to CAIX under hypoxia in vivo. This compound may therefore be used as a fluorescent probe in hypoxic tumour imaging. Compound 2 belongs to a class of positively charged, membrane-impermeable compounds. Therefore, as such compounds do not inhibit intracellular CAs, they may exhibit less side-effects as compared with the presently available compounds (such as acetazolamide), which indiscriminately inhibit all CAs [1,2].

In summary, biochemical, physiological and pharmacological data indicate that inhibition of the tumour-associated CA isozyme IX may be useful in the management of hypoxic tumours that do not respond to classical chemo- and radiotherapy. Therefore, the use of the CA inhibitors described above provide
possibilities of developing both diagnostic tools for the noninvasive imaging of these tumours, as well as therapeutic agents which probably perturb the extratumoural acidification in which CAIX is involved. Many types of highly effective \textit{in vitro} CAIX inhibitors have been developed and evaluated \textit{in vitro}. Whereas many derivatives with enhanced affinity for the tumour-associated isozyme IX over the ubiquitously expressed CAI and CAII isozymes have been discovered, further studies are warranted to understand the behaviour of such compounds \textit{in vivo}, in cell cultures or in animal models of the disease.

CONFLICT OF INTEREST

CS is a Patent Inventor.

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Abbreviations: CA, carbonic anhydrase; HIF, hypoxia-inducible factor; VHL, von Hippel-Lindau; pH, extracellular pH.
Immunohistochemical expression of carbonic anhydrase IX assessed over time and during treatment in renal cell carcinoma

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INTRODUCTION
Carbonic anhydrase IX (CAIX) has been extensively investigated as a prognostic and predictive marker in localized and metastatic RCC. The first studies published showed high CAIX to be related to favourable overall survival [1,2], whereas two recent studies failed to confirm the role of CAIX as an independent prognostic factor [3,4]. Little is known about the impact of treatment and disease progression on the expression of CAIX in RCC. Bui et al. [1] reported CAIX expression to be lower when measured in metastases than the CAIX levels recorded in primary tumours of the same individuals (15 cases), suggesting a loss of CAIX with progression of disease, and in a study by Baldewijns et al. [5] there was a lower expression of CAIX in high-grade compared with low-grade tumours.

In the clinical setting, the collection of serial biopsies from patients with metastatic disease is an ethical and logistical challenge and such material is scant [6]. In addition, in localized RCC most patients have surgery and diagnostic biopsies are only infrequently taken. Therefore, in general, limited material is available allowing for investigations of immunohistochemical markers over time in untreated patients as well as in patients undergoing treatment.

The aim of the present study was to investigate expression of CAIX in repeated tumour biopsies from patients having surgery alone and a group of patients treated with interleukin 2 (IL-2)-based therapy.

PATIENTS AND METHODS
The surgical cohort consisted of a retrospective material of 156 patients treated with radical nephrectomy at the Department of Urology, Aarhus University Hospital, Skejby, from 1992 to 2001. Among these, 57 had diagnostic tumour biopsies taken before surgery, but 31 were fine-needle biopsies and 12 had insufficient material, leaving 14 patients with a matched pair of before surgery biopsy and two routine fixed surgical specimens.

The immunotherapy cohort consisted of 120 patients treated with IL-2-based immunotherapy. All patients were prospectively enrolled in phase II trials and all had low- or intermediate-dose IL-2 with or without interferon α (IFN-α) and histamine-dihydrochloride [7]. Paraffin-embedded baseline and on-treatment tumour biopsy material was available from 52 of these 120 patients. Among the 52 patients, 23 patients had three biopsies evaluable and 29 had two evaluable biopsies. The core-needle biopsies (18 G cutting-needle) were collected by standard ultrasound-guided procedures. Biopsies were taken before treatment (baseline) and at predefined time points according to the treatment schedule (on treatment), to coincide with the outpatient visits. As the biopsies derive from different treatment schedules, the time points vary slightly. The first, second and third biopsy was always taken from the same tumour site, either the primary tumour or a metastatic lesion. All studies were conducted in accordance with the Helsinki declaration II and approved by the local ethics committee and the Danish Medical Agency.

The paraffin-embedded core-needle biopsies were cut in sections (2 μm) and mounted on glass slides. Immunohistochemical staining was done using the mouse monoclonal antibody, MN75 (dilution 1:1000) [8]. In brief, after deparaffinization and hydration the sections were incubated with 0.5% H2O2 for 20 min to block endogenous peroxidase, and subjected to antigen retrieval by microwave cooking for 3 × 5 min in TEG-buffer, pH 9.0. The sections were incubated with protein block serum-free (DAKO X0909; Glostrup, Denmark) followed by incubation with primary antibody overnight. After rinsing in Tris/PBS and Tween washing buffer (0.05%), the Envision System (DAKO K4001) was used as secondary antibody and staining was visualized with diaminobenzidine substrate.Slides were counterstained with haematoxylin, dehydrated and mounted.

For grading of CAIX, the whole biopsy specimen was assessed at ×40. The staining was recorded as a percentage of the total number of tumour cells with positive membranous CAIX expression. The staining intensity was not scored. Two observers (H.K.J., N.M.) evaluated the staining results independently. In case of divergence, a final consensus was reached at a conference microscope. The interobserver variability was very low (Spearman’s ρ = 0.9, P < 0.001).

For comparisons between the different groups of biopsies nonparametric tests were used (Mann–Whitney, Wilcoxon, Kruskall–Wallis and Friedman tests) and for correlations between changes in expression and length of time between the biopsies Spearman’s rank correlation was used. Fisher’s exact test was used for analyses of CAIX using a predefined threshold (high expression: CAIX expression >85% and low expression: CAIX ≤ 85%) used for outcome analyses [1].

RESULTS
In the surgical cohort 14 patients had radical nephrectomy. Of these, two had M+ disease,
six had stage III and six had stage I disease. Twelve had conventional clear cell histology (86%) and two were of the chromophobe subtype (14%).

In the immunotherapy cohort all pts. had M+ disease. Forty-six patients (88%) had clear cell histology, five (10%) had papillary and one (2%) had chromophobe histology.

CAIX IN DIAGNOSTIC BIOPSIES COMPARED WITH SURGICAL SPECIMENS

In the surgical cohort (14 cases) the median (range) expression of CAIX in the diagnostic biopsies was 99 (0–100), as compared with the corresponding two sets of surgical specimens with a median of 99 (0–100) and 99.5 (0–100), respectively. Hence, there was no statistical difference between the three groups of tumour specimens (P = 0.9).

Only one patient had different values in the two surgical specimens (95% and 65%, respectively) compared with a value of 80% in the diagnostic biopsy. The baseline biopsies were collected with a median (range) of 28 (4–55) days before surgery. There was no influence of the length of time between the diagnostic biopsy and surgery and change in expression between the two specimens (Spearman’s ρ = 0.03, P = 0.9), Fig. 1A.

CAIX DURING TREATMENT

In the immunotherapy cohort (N = 52) the sample medians of the baseline biopsies, the second biopsy and in the third biopsy were 98, 98 and 100, respectively (range 0–100). As shown in Fig. 1B,C some of the individual tumours had varying expressions of CAIX during treatment; three had increasing expression, seven had decreasing expression and three of the tumours in Fig. 1C were both increasing and decreasing at the different time points. Assessing the tumours as a group, there was no difference between the expressions of CAIX in the first and second biopsies and also no difference in a matched comparison of all three biopsies (P = 0.3 in the group of two biopsies and P = 0.5 in the group with three biopsies). When comparing the patients treated with IFN-α (N = 17) and without IFN-α (N = 35), there was no difference in change in expression (P = 0.4). The baseline biopsy was taken at a median (range) of 11 (0–94) days before treatment initiation. The median time between treatment initiation and the second biopsy was 15 (12–53) days and for the third biopsy it was 50 (49–133) days. There was no correlation between the length of time from treatment start to the biopsy procedure for the change in CAIX (Spearman’s ρ = −0.08; P = 0.95), Fig. 1B,C. In Fig. 2 examples of CAIX immuno-staining are shown.

CAIX IN PRIMARY TUMOURS AND MASTASES

When comparing these 66 baseline tumour specimens, there was a trend towards a lower value of CAIX in metastatic lesions (median 90%, range 0–100, N = 24) compared with
primary lesions (median 100%, range 0–100, \( N = 42 \)), but it did not reach statistical significance \( P = 0.1 \). CAIX expression in patients with localized disease \( (N = 12) \) was not different from CAIX in pts. with metastatic disease \( (N = 54) \) \( P = 0.4 \). Five patients had biopsies from both primary tumours and metastases. In these cases, the expression of CAIX was similar over time, with few changes in expression in both primary tumours and metastases (Table 1).

### CAIX Expression over Time and During Treatment in RCC

CAIX expression was consistent in matched diagnostic biopsies and surgical specimens. In two separate surgical specimens from each tumour, we also observed equal expression, with only one tumour having different expression. In a study by Leibovich et al. [4] intratumour heterogeneity was addressed for CAIX staining intensity and 55.8% of the tumours had different staining intensities. We assessed the extent of CAIX-positive staining and this was very homogenous. We did not assess intensity as it is known to be sensitive to slide thickness and day-to-day variation in staining procedures.

The present study specifically addressed short-term changes in CAIX in RCC after IL-2-based treatment. Focusing on the overall change in CAIX for all biopsies as a group there was no impact of treatment on the expression of CAIX. However, focusing on individual tumours some changed after treatment, with no specific trend in any direction. The reason for the change in individual tumours is not known; preclinical studies have shown IFN to cause an increase in CAIX/G250 [9], but in the present data set there was no association between increasing CAIX values and administration of IFN-\( \alpha \).

Another theory is that up-regulated CAIX is regarded as non-self by the immune system resulting in destruction of CAIX-positive cells.

### Discussion

The present study supports data on CAIX being a robust immunohistochemical marker in RCC with little influence of surgery or intervention by IL-2-based immunotherapy. The study also verifies the difficult task of collecting tumour material from patients with cancer, both pro- and retrospectively, starting with a total of 276 patients and resulting with sufficient serial tumour material from only 66 patients. Although the total sample size is small, it represents a high number for studies of this kind.

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Another theory is that up-regulated CAIX is regarded as non-self by the immune system resulting in destruction of CAIX-positive cells.
[10], leading to a selecting of the CAIX-negative cells and therefore a decrease in the CAIX level. The association between good prognosis and high levels of CAIX supports this hypothesis. These different theories might reflect the large variety of functions of CAIX, serving as a tumour antigen, a marker of progression and a marker of hypoxia [11].

In the total set of tumour samples, we compared the expression of CAIX in biopsies taken from primary tumours and from metastases and there was a trend towards a lower value in the metastases, but it did not reach statistical significance. In addition, there was no association between disease stage and expression of CAIX. However, only 14 patients had localized disease, which could explain the lack of association. In a direct comparison of five matching primary tumours and metastases, there was no association between disease progression and a marker of hypoxia [11].

Interestingly, tumours initially identified as either high- or low CAIX expression based on the previously defined threshold of 85% [1], seem in general to remain stable during treatment, favouring the continuous use of this threshold for CAIX as a prognostic marker in RCC.

In conclusion, the present study supports data on CAIX as a valid immunohistochemical marker in RCC with little influence of surgery or IL-2 treatment. Further studies are needed to clarify any prognostic impact of changes in CAIX and the impact of treatment regimens targeting CAIX more directly.

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CONFLICT OF INTEREST

All authors declare no conflict of interests.

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Abbreviations: CA, carbonic anhydrase; IL-2, interleukin 2; IFN, interferon.
INTRODUCTION

It has been estimated that bladder cancer will affect 67,160 Americans in 2007 and that 17,120 will die of the disease [1]. The number of newly diagnosed cases has been increasing over the past decade, mainly due to more intensive evaluation of patients presenting with haematuria and irritative voiding symptoms, and improved physician and patient education [2]. The median age of newly diagnosed patients is 73 years, with men having a three-fold higher lifetime risk of developing bladder cancer than women [3]. About 90% of all bladder cancers are TCCs [4], and ≈70% of these tumours will be diagnosed at a noninvasive tumour stage (Ta, Tis, or T1). Despite aggressive treatment with a combination of transurethral resection (TUR) and intravesical agents such as BCG or mitomycin, recurrence rates are 50–70%. Furthermore, progression into muscle-invasive disease develops in 10–20% of patients who initially present with noninvasive tumours TCC [4]. Currently, there are few prognostic factors for the prediction of TCC recurrence or progression. Thus far, these factors are mainly clinical, and include stage, grade, tumour size, number of tumours, and associated carcinoma in situ (CIS). However, prediction of these endpoints remains difficult and integration of molecular markers such as p53 may be beneficial, but studies remain inconclusive [5].

Hypoxia is a common consequence of rapid growth of many vascular tumours and is an important regulator of gene expression. Carbonic anhydrase IX (CAIX) protein, an hypoxia-inducible member of the CA family that regulates intracellular pH during periods of hypoxia, is thought to play a role in the regulation of cell proliferation, cell adhesion, and tumour progression [6,7]. This brief review summarizes the current knowledge on CAIX expression in bladder TCC and its potential role as a marker of prognosis and as a therapeutic target.

GENERAL ROLE OF CAIX IN CANCER

CAs are a large family of zinc metalloenzymes that are found in almost every organism. Today, there are 15 members of this family known, which differ with regards to tissue distribution and subcellular localization [7,8]. CAs catalyse the reversible reaction \( H_2O + CO_2 \leftrightarrow H^+ + HCO_3^- \), which is crucial to a wide variety of processes including pH regulation.

CAIX belongs to the group of membrane-associated CAs. The term CA9 refers to the corresponding gene, which is located on chromosome 9p12–13 and consists of 1609 base pairs arranged in 11 exons. CA9 encodes for the 459 amino acid CAIX protein. CA9 is one of the 50 genes that are up-regulated by hypoxia-inducible factor 1α (HIF-1α). Therefore, a binding site for HIF-1α/hypoxia-responsive element is present in the CA9 promoter.

CAIX is not expressed in most benign organs and tissues. There is weak expression in the gastric mucosa, small intestine, biliary tract and seminal ducts [9]. However, CAIX is abundantly expressed as a direct consequence of hypoxia in numerous cancers [9]. In many of these cancers, the greatest staining intensities are on luminal surfaces or on surrounding areas of necrosis. Clear cell RCC is an exception to this general rule, and is the only cancer that shows a uniform staining pattern suggesting, probably that its expression in RCC is not a result of tumoral hypoxia but of a constitutively up-regulated HIF-pathway secondary to the inactivation of the von Hippel-Lindau (VHL) tumour suppressor gene [6]. Studies show that high CAIX expression yields an aggressive tumour phenotype and poor prognosis in breast cancer [10], cervical cancer [11,12], non-small cell lung cancer [13], soft tissue sarcoma [14] and adenocarcinoma of the upper gastrointestinal tract [15,16], while the opposite has been noted in RCC [17,18].

EXPRESSION IN BLADDER CANCER

Normal urothelial tissue [19,20] does not express CAIX. By contrast, expression is observed in 70–90% of TCCs [20,21], but rarely found in CIS. In 10 cases of CIS studied by Turner et al. [19], expression was weak in three and absent in seven of the specimens. In patients with metastatic TCC, simultaneously extirpated metastases show higher CAIX expression than the corresponding primary tumours [20].

Although expressed by most TCCs, expression frequency and intensity are usually low. Tumours stained by Hoskin et al. [22] showed an average stained tumour fraction of only 9%. Wykoff et al. [23] studied 14 TCCs derived from patients who had received pimonidazole before surgical excision. Although 12 of 14 specimens showed CAIX expression, the median percentage of tumour cells staining positive was only 5%. The authors compared expression levels with those of the bioreductive hypoxia marker pimonidazole. There was significant correlation between both markers, although expression was less strong for CAIX [23]. A similar relationship has been shown for CAIX and vascular endothelial growth factor (VEGF)-A mRNA levels [19].

Staining of CAIX is heterogeneous throughout each tumour [22]. Maximum staining is on the luminal surface of the papillary structures (Fig. 1). Additionally, CAIX expression is seen around areas of necrosis in invasive tumours or metastases (Fig. 2) [19,22,23].

Studies suggest that CAIX is expressed in relation to stage and grade. Ord et al. [24] found that 13 out of 21 of the noninvasive, but only 3 out of 11 of the invasive tumours stained positive for CAIX. More recently, in a series of 98 patients with bladder cancer, the same group reported an increase in CAIX positivity among T1, T2, and T3 tumours, but expression levels decreased in T4 [25].

Our recent data shows that CAIX might be differentially regulated in low-grade and
high-grade TCC [20]. In a series of 522 TCCs, 0% of normal urothelial samples, 85% of the grade 1 tumours, 79% of the grade 2 tumours, but only 63% of the grade 3 tumours stained positive for CAIX (P < 0.001). In contrast to our findings, one group did not show a significant relationship between CAIX expression and grade [25]. Overall, published data supports a role of CAIX as a diagnostic marker for TCC. CAIX might complement urinary cytology as a noninvasive marker to monitor for TCC because it is able to differentiate between normal urothelial cells and low-grade tumours. For example, in a small series of cytological samples, CAIX staining of urinary sediment could distinguish between benign papillary clusters and low-grade papillary tumours.

**ROLE AS A PROGNOSTIC FACTOR**

Several studies on the role of CAIX as a prognostic factor in bladder cancer have been inconclusive. Most studies did not show a significant relationship, but all were limited by few samples and subsequent low statistical power. A large recent study from the authors’ institution showed overwhelming prognostic significance in both superficial and muscle-invasive disease [20].

Ord et al. [25] reported on the role of hypoxia and necrosis in 98 patients with bladder cancer treated by cystectomy. The authors evaluated staining of CAIX, HIF-1α, HIF-2α, and Bcl2/adenovirus EIB 19 kDaA interacting protein 3 by immunohistochemistry. No association was found between CAIX and survival (P = 0.55). Moreover, none of these markers were retained as independent prognostic factors in multivariate analysis.

In a series of 49 cases reported by Turner et al. [19], recurrence- and progression-free survival in patients with low and high CAIX expression was similar. The authors correlated mRNA expression of VEGF-A and immunohistochemical expression of CAIX. Although there was a striking overlap, VEGF-A expression, but not CAIX, was predictive of time to recurrence and risk of stage progression.

Hussain et al. [26] investigated 57 patients with newly diagnosed TCC for CAIX expression and correlated their findings with survival. Tumours expressing CAIX weakly showed a trend towards shorter survival (P = 0.21). Stratified by stage, no significant association was found between CAIX and survival in superficial (P = 0.9) or invasive TCC (P = 0.94).

Hoskin et al. [22] studied CAIX expression in relation to survival in 64 patients treated by radiotherapy with carbogen and nicotinamide. Higher CAIX expression predicted worsened cancer-specific and overall survival. The 5-year overall survival rate for tumours expressing higher than the median CAIX value was 35% compared with 71% with low levels. Furthermore, CAIX expression was an independent prognostic factor in multivariate analysis (hazard ratio 3.21, 95% CI 1.16–10.22, P = 0.02), when CAIX and GLUT-1 expression were entered individually. However, the significance was lost when both variables were entered simultaneously.

Recently, we reported on survival of 351 patients undergoing surgical resection for bladder cancer [20]. For patients with Ta TCC undergoing TUR, higher CAIX expression (>45%) was associated with poorer recurrence-free survival (P = 0.02). In patients with T1 tumours, higher CAIX expression (>20%) also conveyed a worse prognosis with respect to recurrence-free (P < 0.001) and progression-free survival (P = 0.01). In patients undergoing cystectomy for muscle-invasive TCC, higher CAIX expression (>10%) was an independent prognostic factor of diminished overall survival (P = 0.002) [20]. Taken together, CAIX has been identified as an important predictor of the three survival endpoints in bladder cancer: recurrence, progression, and overall survival.

**ROLE AS A POTENTIAL THERAPEUTIC TARGET**

Cornerstones in the treatment of bladder TCC include surgery, intravesical instillation, and systemic chemotherapy with or without radiation. As CAIX is expressed in 70–90% of TCCs, but not in normal urothelial tissue, it represents a potential cancer-specific therapeutic target. First, particularly as CAIX is expressed on the luminal cell surface of the tumours, there may be a role as an intravesical-targeted agent for instillation therapy. For this purpose, baseline immunohistochemical staining of CAIX expression levels could be used to select patients and to determine whether the tumour can be targeted appropriately with a CAIX-directed approach. Second, there may be a role in systemic treatment for patients with metastatic disease. The chimeric monoclonal antibody G250, for example, has been extensively evaluated clinically in patients with RCC [27,28]. It was well-tolerated and showed promising antitumour effects [27]. A large randomized trial in an adjuvant setting is currently under way [29].

Recently, antibody-drug conjugates have gained attention for the treatment of advanced cancers [30]. In this concept, monoclonal antibodies are linked with cytotoxic agents, which specifically bind to target cells that express the antigen. Using this approach, the potential cytotoxic effect could be limited through targeted tumour delivery. For the treatment of bladder cancer, for example, G250 could be conjugated with a toxin or with a chemotherapeutic drug such as cisplatin.

Moreover, vaccine therapies have shown promise of efficacy in RCC [31–33]. Given
that bladder cancer represents an immunosensitive disease responding to agents such as interferon α and BCG, administration of vaccines may also be an effective approach. Taken together, the role of CAIX as a therapeutic target in TCC is conceptually sound and warrants further investigation.

CONCLUSIONS

CAIX is a bladder cancer-specific antigen that is not expressed in normal urothelial tissue but is expressed in 70–90% of TCCs. Expression is usually heterogeneous, with maximum staining seen on the luminal surfaces of the papillae and in perinecrotic areas. It appears that expression levels are related to stage and grade. Studies indicate that higher CAIX expression is associated with adverse prognostic features, such as increased recurrence and progression, and worse survival. CAIX may be exploited as a therapeutic target for both intravesical and systemic treatment. Thus, CAIX has important tripartite implications as a diagnostic, prognostic and therapeutic molecular marker of bladder cancer.

CONFLICT OF INTERESTS

All authors declare no conflict of interests.

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Abbreviations: TUR, transurethral resection; CIS, carcinoma in situ; CA, carbonic anhydrase; HIF, hypoxia-inducible factor; VHL, von Hippel-Lindau; VEGF, vascular endothelial growth factor.
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c Text, sub-divided into:
   introduction
   subjects/patients (or materials) and methods
   results
d Legends to illustrations
e Tables and their legends

Please ensure that any Tables and Figures are cited in the text.

Points of Technique and Case Reports are no longer accepted for publication in the Journal. Please note that any point of techniques or case reports submitted will be returned immediately to the sender.

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Surgery Illustrated. Submissions to Focus on Details are welcome. This second part of "Surgery Illustrated" intends to highlight one surgical step with one or two figures and a brief description. Articles must not be related to a surgical procedure previously published in the "Surgical Atlas". Let us know if you have any questions in this regard.

Manuscripts highlighting one step of a surgical procedure (e.g. specific instrument or surgical trick) should be no more than two or three pages and contain a maximum of two illustrations.

Material for the illustrator:

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• The text should be structured as follows:
  • Short introductory paragraph describing the problem
  • Description of technique as legends to figures

Summary, including benefits over standard surgical step, shortcomings, possible complications and troubleshooting.

If accepted for publication, all illustrations will be redone to conform with the format and appearance of Surgery Illustrated.

Submissions to Focus on Details should be directed to:
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