Current Topics in Biochemical Research
Vol. 15, *2*, 2013

Review

Regulation and role of carbonic anhydrase IX and use as a biomarker and therapeutic target in cancer

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ABSTRACT

In many solid tumors the rapidly proliferating cancer cells create an environment with insufficient oxygen supply. In response to this tumor hypoxia, cancer cells adapt gene expression to thrive in the altered microenvironment. One of these adaptations is the shift towards anaerobic glycolysis. Lactic acid accumulates hand-in-hand with carbon dioxide, leading to acidification of the extracellular environment. Carbonic anhydrase IX (CAIX) is one of the expressed genes required in response to hypoxia for cell survival, as its role in intracellular pH maintenance allows the cancer cells to adapt to the extracellular environment. In addition, CAIX stimulates the migratory pathways of cancer cells and the aggressive/invasive phenotype of tumors, and has been shown to play a key role in signaling cascades acting similar to a feed-forward regulator of its own expression. Hence CAIX expression in several tumor tissues indicates its relevance as a general marker of tumor hypoxia. Moreover, its expression is correlated to poor clinical outcome in several tumor types. In this review we describe the biochemical characteristics of CAIX and the regulatory pathways of its production. In addition we summarize the CAIX expression patterns in normal and cancerous tissues. Lastly, we discuss the physiological role of CAIX in the tumor microenvironment in terms of pH regulation, proliferation, cell motility, adhesion, invasiveness, and signaling, and how this identifies CAIX as an anti-cancer target.

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KEYWORDS: carbonic anhydrase IX, hypoxia, pH homeostasis, metastatic cascade, hypoxic-inducible factor-1α, CAIX mimic, prognostic marker, therapeutic resistance

INTRODUCTION

It has been well established that hypoxia in various solid tumors is associated with poor prognosis and survival rates in cancer patients [1]. Hypoxia in the tumor milieu is typically defined by a reduction in overall O_2 content ($\leq$ 1.0%) and a decrease in extracellular pH (~6.5) [1, 2]. This microenvironment acidification alters the tumor milieu resulting in a resistance to common anti-cancer treatments such as radiation and chemotherapy. This occurs due to a lack of available ionizable oxygen species and reduced permeability of chemotherapeutics into avascular areas [1, 2]. Furthermore, induction of hypoxia in tumor cells has been associated with tumor cell proliferation, angiogenesis, cell motility, invasiveness and overall tumor cell survival, which ultimately lead to malignancy [1]. The induction of hypoxic conditions in tumor cells has been classified by a phenomenon known as the Warburg effect, where by proliferating cancer cells utilize glycolysis as the predominant mode of energy production resulting in an increased cellular export of lactic acid and protons thus attributing to a reduction in extracellular pH (pHe) [3, 4]. It has been shown that this modulation of tumor cell metabolism is directly associated with upregulation of hypoxia-inducible factors (HIFs) [5]. Specifically, HIF-1 upregulation induces activation of genes associated with glycolytic metabolism, angiogenesis, cell motility, and also inflammation [5, 6].

A major aspect of tumor cell survival is the ability to thrive in the hypoxic-stress induced microenvironment. To do so, tumor cells must maintain an intracellular pH (pHi) at near physiological levels in order to continue essential metabolic functions allowing for proliferation. Furthermore, tumor cells can utilize the acidic pHe, to induce cell migration and invasive properties, a key characteristic of tumor metastasis [6, 7]. Maintenance of pH homeostasis in tumor cells is complex with several mechanisms regulating the differential pHi/pHe gradient. Arguably the most important is carbonic anhydrase (CA) activity [6, 7]. In mammals 16 different isoforms of carbonic anhydrase have been identified and categorized as the α-class [8, 9]. All isoforms in the α-class are homologous metalloenzymes that catalyze the reversible hydration of CO_2 to HCO_3^- [8, 9]. This occurs by way of a double-displacement mechanism initiated by nucleophillic attack of CO_2 (Eq.1) via a tetrahedrally coordinated zinc-hydroxide [9, 10]. Upon conversion of CO_2, an H^+ is released into the cellular environment (B) via a transferring mechanism (Eq. 2) between the active site solvent and the imidazole group of a proton shuttling histidine residue towards the surface of the enzyme [10].

$$\text{Eq 1.} \quad E{:}Zn\text{-}OH^- + CO_2 \leftrightarrow EZnHCO_3^- \overset{H_2O}{\leftrightarrow} E{:}Zn\text{-}H_2O + HCO_3^-$$

$$\text{Eq 2.} \quad E{:}Zn\text{-}H_2O + B \leftrightarrow E{:}ZnOH^- + BH^+$$

Of the 16 mammalian isoforms of CA, human isoforms IX and XII (CAIX, and CAXII) have been identified as tumor associated. Both CAIX and CAXII are extracellular membrane-bound isoforms of CA and show relatively high-expression in solid tumors with contrasting low expression in normal tissues [7, 11]. Additionally, CAIX and CAXII have been found to contribute to tumor metastasis, therapeutic resistance, and overall bad prognosis in cancer patients [6, 7]. However CAIX has been determined to be more prevalent in terms of expression in hypoxic tumors, such that its expression levels show strong correlation with HIF elements [11-13]. As such CAIX has also shown to play a critical role in metabolic modulation of tumor cell growth, proliferation, migration, adhesion, and cell survival [7, 14, 15]. CAIX has also shown to have a functional role in cell-signaling pathways in tumor cells [16]. High levels of CAIX in aggressive cancerous tissue in combination with identification of its essential roles in various physiological processes of tumor cells have defined CAIX as an important biomarker and therapeutic target.

In this review we describe the biochemical characteristics of CAIX and regulatory pathways of its production [6, 7]. In addition we discuss CAIX expression patterns in normal tissue and cancers, and how this relates to poor prognosis in cancer patients [6, 7, 17-19]. Lastly, we describe the physiological role of CAIX in the tumor microenvironment in terms of pH regulation, proliferation, cell motility, adhesion, invasiveness, and signaling, and how this identifies CAIX as an anti-cancer target.

Biochemical and biophysical characterizations of CAIX

CAIX is a transmembrane glycoprotein that was first discovered in HeLa cells. Initially CAIX was labeled as a MN-protein despite showing CA activity [20]. The *CA9* gene encoding for the enzyme is 10.9 kb in length, and has been mapped to chromosome 9p12-13 [21, 22]. Full-length *CA9* contains 11 exon-coding regions that translate to 459 aa [21, 22]. The construct of CAIX (Figure 1A) can be divided into a 414 aa N-terminal extracellular domain, a 20 aa transmembrane domain (TM), and a 25 aa C-terminal intracellular domain (IC) [23, 24]. The extracellular domain consists of a 37 aa signal peptide, a 59 aa proteoglycan-like domain (PG), and a 257 aa catalytic domain (CA)

that is structurally homologous to the other isoforms in the mammalian CA class [21, 24]. Variations of the *CA9* gene exist due to alternative splicing mechanism that have been shown to produce a truncated version (lacking TM and IC domains) of CAIX implicating its importance in signaling [25]. The presence of the PG domain (59 aa), which has been named due to its resemblance to the keratin sulfate attachment domain of human aggrecan, is a unique feature of CAIX compared to other isoforms. The PG domain has been proposed to play a crucial role in cell adhesion and catalytic activity *in vitro* [26, 27]. Mass spectroscopic analysis of mature CAIX indicates that the enzyme is stabilized by an intramolecular disulfide bond between Cys119-Cys299, and also contains two unique *N*-linked and *O*-linked glycosylation sites at Asn309 and Thr78, respectively [27]. The total MW of monomeric CAIX has been estimated at 49.7 kDa, but migrates in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (non-reducing conditions) and western blots as a double band between 54/58 kDa. This effect is most likely due to glycosylation and/or equivalent populations of mature and pro-forms of the enzyme with both possessing a propensity to oligmerize in non-reducing conditions [20, 23, 24, 27]. Alternatively, a soluble form of CAIX that consist of the PG and CA domains only (s-CAIX) has been observed in culture medium and in the sera of various cancer patients [28]. This s-CAIX has been shown to migrate at 50/54 kDa on western blots and has been proposed to form via proteolytic cleavage of the extracellular domains, or as per the alternative splicing mechanism mentioned previously [25, 28]. It has further been established by MS and X-ray crystallography data of the CA domain, that CAIX exists as a homodimer (Figure 1B) with intermolecular disulfide bridges forming between adjacent Cys137 of the mature enzyme [23, 24, 27].

Structural alignments between the CAIX CA domain and CA II display high structural conservation between catalytic sites with only slight differences occurring between the hydrophobic, and hydrophilic clefts [24-27]. This high homology between isoforms leads to difficulties when designing CAIX specific inhibitors, as CA II is ubiquitously expressed in normal tissue [6-9]. Naturally, due to the high conservation between active sites, the catalytic efficiencies between CAIX and CA II are comparable. O^{18}-exchange measured by mass spectrometry show that CA II and the CA domain of CAIX have similar k_{cat}/K_m values of 100 μM^{-1} s^{-1} and 55 μM^{-1} s^{-1}, respectively [10, 29]. In addition the CA domain of CAIX also exhibits a similar pH profile as that of other CA isoforms. This is deduced from an inflexion point with a pKa value of 7.01 for CAIX compared to that of 6.90 and 7.10 for CA I and II, respectively [23-30]. Alternatively, when CAIX contains both PG and CA domains there is a shift in pKa to 6.49, which is consistent with the typical pH of the hypoxic tumor milieu [5, 23, 24]. This observation has led to the hypothesis that the PG domain acts as an internal buffer, such that the catalytic efficiency of CAIX is not hindered from the low pHe of the hypoxic tumor microenvironment [23, 24]. However this observation has only been seen *in vitro,* and recent data obtained from measuring CAIX activity in breast cancer cells show that the kinetic profiles of native CAIX *in vivo* compared to the truncated form of CAIX (CA domain only) are indistinguishable, hence disputing the PG domain's influence on catalytic efficiency [30, 31]. Phosphorylation sites have also been identified on the IC domain of CAIX corresponding to Thr443, Ser448, and Tyr449 of the full length species that have been shown to modulate catalytic activity and also play a role in the PI-3K/Akt signaling cascade [32, 33].

Hypoxia-inducible factors modulate CAIX expression

Hypoxia-inducible factors (HIFs) are major regulators of tumor biology and are directly associated with increased metastatic potential and chemo- and radio-insensitivity in solid tumors [5, 33]. HIF-1, which is predominantly associated with aggressive tumor cells, consists of a heterodimeric complex of ubiquitously expressed α- and β-subunits (HIF-α and HIF-β) [33]. Formation of the HIF-1 complex mediates a transcriptional response to hypoxic stress in both normal and neoplastic tissue by interacting with target genes known as hypoxia-response elements (HREs) [5]. HIF-β, also known as aryl hydrocarbon receptor nuclear translocator (ARNT), is maintained at a constant level in both normal and neoplastic tissue [5]. Alternatively, HIF-α expression is strictly regulated and hence the

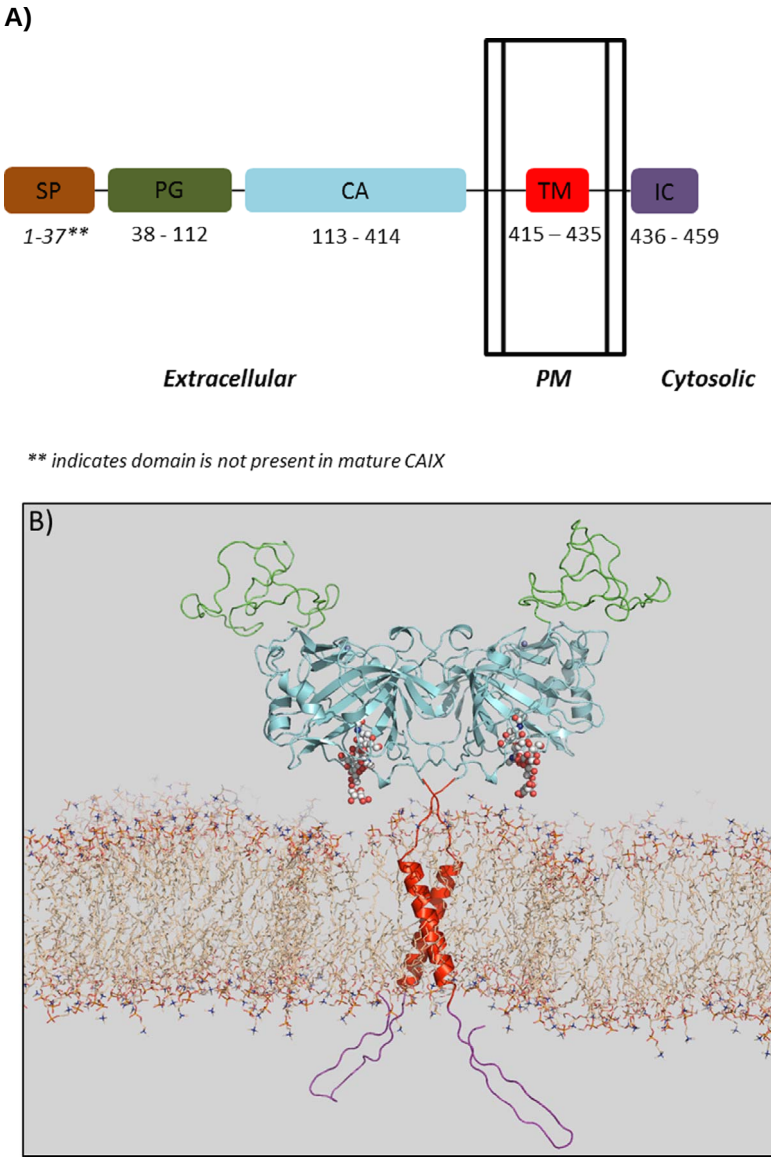

Figure 1. CAIX Structure. A) Schematic diagram of the structural arrangement of the CAIX domains. These include the signal peptide (SP, brown) that is not present in mature CAIX, a proteoglycan-like domain (PG, green), catalytic domain (CA, pale cyan) that displays high homology with other mammalian isoforms, a transmembrane anchor (TM, red), and intracellular domain (IC, purple). B) Structural model of the homodimeric CAIX as it is located in the cell membrane. The extracellular facing CA and PG domains (pale cyan and green, respectively) are anchored by a ~20 residues TM domain (red) that is predicted to form a helix-helix interaction within the lipid-bilayer. The existence of an N-terminally linked proteoglycan-like extension is a unique feature of CAIX that is predicted to be involved in catalysis and cellular adhesion. In this model the PG domain exhibits no secondary structure and is displayed as a random coil (as predicted by *Robetta*). Also, unlike several mammalian isoforms of CA, CAIX contains both *N*- and *O*-linked glycosylation sites at Asn309 (shown as spheres) and Thr78 (not shown), respectively. The IC domain of CAIX has been proposed to be involved in cell signaling pathways and is predicted to exist as an unstructured loop. (The CA domain of model was made using coordinates from PDB: 3IAI. The PG domain was created using structure prediction server *Robetta*, and *Chimera* and *COOT* software packages were utilized for generation of the lipid bilayer, TM and IC domains, and positioning of each domain to obtain rigid body coordinates for the final model. The final figure was made using *Pymol*. This model was generated based on observations from [23, 24, 26, 27, 32, 33]).

mediating step in HRE activation [5]. HIF-α subunits exist as three isoforms 1, 2 and 3 with HIF-1α predominantly associated with hypoxia mediated cell proliferation, angiogenesis, chemo- and radiation resistance, and inflammation in tumor cells [34, 35]. HIF-1α is positively regulated by environmentally low levels of O_2 [36]. In normoxic conditions HIF-1α subunits are hydroxylated at two proline residues by HIF-specific prolyl hydroxylase (PDH) [5, 37]. Proline hydroxylation by PDH induces HIF-1α ubiquitination by von Hippel Lindau tumor suppressor protein (VHL) that leads to proteosomal degradation [38]. HIF-1α expression is also mediated by hypoxia-independent oncogenic signaling via phosphoinositide-3 kinase (PI-3K) Akt-mammalian target of rapamycin (mTOR) pathway [37-39]. Alternatively, this process is down regulated by ADK/AMPK-dependent inactivation of the mTOR signaling pathway [37-39].

HIF-1α mediated HRE binding causes activation of >800 genes [40]. The activated genes include vascular epidermal growth factor (VEGF) which induces angiogenesis, glucose transporter-1 (GLUT-1), which increases glycolytic metabolism via import of usable glucose, insulin-like growth factor-2 (IGF2) that provides apoptotic resistance, ATP-binding cassette transporter B1 (ABCB1), which causes chemotherapeutic resistance, and CAIX [5, 40-44]. Additionally, activation of HRE causes an upregulation of migratory and invasive elements that contribute to metastatic behavior of tumor cells. Contributing factors to this specific phenotype are collagen type V-α1 (COL5A1) that regulates the formation of extracellular matrix (ECM), and several elements that degrade the ECM including metalloproteinases (MMPs), urokinase-type plasminogen-activator receptor (suPAR), and cathepsins [40-45]. CAIX, which is mediated via HIF-1α, has shown to be directly correlated and play an essential role in the aforementioned functions in the tumor cell [7, 45].

Normal and neoplastic expression patterns of CAIX

As mentioned previously CAIX expression is directly dependent on HIF-1 activation [5, 45]. As such CAIX expression has been observed to be regulated by variations in this pathway relative to certain normal and neoplastic tissues [5-7, 46]. More importantly CAIX expression in normal tissue remains limited to certain epithelial tissues of the gut, and reproductive tracts, whereas its expression is ubiquitous in a range of highly metastatic tumor tissues [6, 46]. As a result CAIX has been established as a prognostic biomarker for various malignancies [6, 46]. In the next section we explore the expression patterns of CAIX in normal and neoplastic tissues relating to its significance as a prognostic tool, and provide a brief overview of some of the regulatory mechanisms that induce expression in each tissue type.

Normal tissue

Immunohistochemical analysis shows that normal expression of CAIX is localized in the human gut. Specifically CAIX expression is observed in the basolateral surfaces of enterocytes of the duodenum, jejunum, and also the ileal mucosa [46]. The most prominent levels of CAIX were shown to be in the proliferating crypt enterocytes, a major component of the intestinal stem cell niche [46, 47]. Proliferation in enterocyte crypts is induced by Wnt-signaling, which is positively correlated with HIF-1α expression. This hints at the possibility of CAIX functioning in intestinal stem cell proliferation and regulation [47-49]. CAIX is also seen in higher abundance in developing embryonic tissues of the lung, gut epithelium, and skeletal muscle, all of which, other than gut epithelium, show decreasing CAIX levels in adult tissue [50]. CAIX expression has also been measured by northern blot and immunohistochemical staining in pancreatobilary epithelium, mesothelial cells, ovarian surface epithelium, choroid plexus basal cells of hair follicle, and also fetal rete testis [50, 51]. This observation indicates that the majority of CAIX expression is associated with microenvrionments of low pH or high rates of cell proliferation suggesting a possible functionality of CAIX as regulatory element in these normal tissues. However there is limited data available to conclude these remarks.

Breast cancer

High levels of CAIX expression have been routinely found in highly aggressive breast cancer tissue despite normal breast epithelium null of CAIX.

In addition benign neoplasms show very low levels (<11%) of expression [52]. High levels of CAIX expression are found in ~50% of all *in situ* breast cancer cases, with the majority being associated with poor prognosis patients [52, 53]. When observing invasive breast mucinous carcinoma cells, CAIX expression is shown to positively correlate with GLUT1 and monocarboxylate transporter-1 (MCT-1) expression, both of which are up-regulated by way of HIF-1α for induction of glycolysis and further establish the hypoxic tumor milieu [54]. This observation typically translates to areas of tumor necrosis in breast cancer patients, which further acts as a key indicator for poor prognosis [55]. *In vivo* activity assays in MDA-MB-231 cells show that CAIX expression and activity can be modulated upon induction of hypoxia. This provides additional evidence that hypoxia-induced CAIX levels are associated with metastatic proliferating tumor cells [31]. In addition high levels of CAIX occur in fibrotic focus points, or scar-like areas consisting of cancer associated fibroblasts (CAFs), that have begun to replace necrotic cells, and act as a predominant marker for invasive cancerous tissue in breast carcinomas [54]. Given that CAIX displays no expression in normal breast epithelium, low levels in benign breast tumors, and high levels in malignant tumors, CAIX has been proposed as marker for both tumor hypoxia, and as a prognostic indicator in breast cancer patients [53-55].

Lung cancer

Similar to cases of breast cancer CAIX expression levels are linked to poor prognosis in lung adenocarcinoma patients. High levels of CAIX expression have shown to be directly correlated with tumor necrosis observed in patients with early-stage non-small cell lung cancer (NSCLC) [56]. Specifically, increased levels of CAIX expression were seen in 72% of NSCLC patients, with the majority showing tumors with increased malignant potential or a high risk of reoccurrence [56]. Also alike breast carcinoma expression patterns of CAIX, lung adenocarcinoma cases show the majority of CAIX expression associated with CAFs [56, 13]. CAIX has also been observed to be positively correlated with MMP-9, VEGF, and SDF-1, common positive indicators of hypoxia and metastatic tumor cells [13, 57]. However expression studies in tumors from stage I/II NSCLC patients have indicated that, in comparison to levels of VEGF and MMP-9, levels of CAIX display the highest dependency on the presence of HIF-1α [57]. This further facilitates the notion that CAIX is tightly regulated by HIF-1α expression and can be exploited as an independent marker for predicting areas of tumor aggressiveness in NSCLC patients [56]. Alternatively, it was shown that CAXII expression, which was previously believed to be associated with highly metastatic lung tumors, was observed to be less prevalent [13, 57]. Interestingly, the results from analyzing NSCLC patients indicated that high CAIX/low CAXII expression was more associated with a high cumulative incidence of relapse and poor overall survival [13]. In essence, this result suggests a reciprocal relationship between the expression of CAIX and CAXII, such that high CAXII levels in NSCLC patients may transition to a more favorable prognosis [13]. The results obtained from these studies poise CAIX to be a valuable and reliable prognostic indicator in lung adenocarcinoma.

Kidney cancer

CAIX levels in Renal Cell Carcinoma (RCC) are tightly regulated by HIF-1α expression [17, 58]. This has been determined by observing that CAIX expression levels are suppressed in the presence of VHL, which negatively regulates HIF-1α levels, to an even greater extent than VEGF [17, 58]. The presence of VHL however, had little effect on CAXII expression, which exhibits intrinsically low levels of expression in normal kidney [58]. Once present, CAIX expression levels can be maintained in RCC tumors at pericellular or mildly hypoxic regions through participation of the EGF stimulated PI-3-Kinase pathway [17]. In this particular case CAIX can act similar to a feed-forward regulatory element of HIF-1α expression through the activation of Akt [17, 36]. As a result CAIX has found direct use as an independent reliable prognostic marker in metastatic RCC patients and has been utilized in Phase III efficacy studies of the anti-cancer drug sorafenib [59, 60]. The capability CAIX has to predict prognosis in progressive metastatic RCC patients

has further prompted the development of a WX-G250, a chimeric monoclonal anti-body specific for CAIX that is combined with low-dose interferon-α (marketed as RENCAREX®, Wilex AG, Munich Germany) to act as an anti-cancer therapeutic [61]. Currently, RENCAREX® has just completed clinical Phase III trials as an adjuvant therapy against clear-cell RCC patients (ccRCC) with results awaiting publication (http://www.wilex.de/portfolio-english/rencarex/phase-iii-ariser/) [61]. In addition, s-CAIX levels have shown promise as a prognostic marker in RCC patients as they have been correlated to tumor progression [28, 62]. Measurements of s-CAIX are attractive as a prognostic indicator to measure post-treatment efficacy since they can be obtained with less invasive techniques [62]. However, s-CAIX levels only remain in the blood within a few days post-treatment and therefore only serve useful if measured within this time frame [28, 62]. Despite the promise CAIX has shown as an independent prognostic marker of RCC, it has been suggested that, CAIX is unable to predict prognosis after long-term follow up in ccRCC patients [63]. Alternative to this data however, the majority of evidence contradicts this observation; hence, CAIX is still considered as an invaluable prognostic indicator.

Colorectal cancer

Transcriptional analysis of colorectal carcinomas (CRC) indicates CAIX expression is directly correlated with a poor grade of tumor differentiation [64]. Furthermore, CAIX expression has been linked to survival rates and poor disease outcome in CRC patients [65]. Not surprisingly, CAIX expression was coupled to that of HIF-1α and positively correlated with GLUT-1 and VEGF expression levels [65, 66]. Similar to the expression patterns in cases of RCC, CAIX expression is equally regulated by both hypoxic-stress induction, and PI-3-Kinase signaling with Akt activation occurring downstream [36, 65]. Akt signaling is a key regulator in tumor progression in CRC and therefore indicates that CAIX may have important functionality in this signaling cascade [17, 36, 65]. It has been shown via utilization of the monoclonal anti-body G250 (derived from RENCAREX®) that CAIX can be used for clinical detection of hypoxia in CRC patients [67]. This result is very promising as it increases the repertoire of the utility of CAIX as a prognostic marker.

Cervical and vulvar cancer

Clinical observations regarding CAIX levels in advanced-staged cervical cancer patients indicate that overexpression of CAIX is directly correlated with aggressive tumor characteristics and disease-specific survival [68, 69]. In relation to HIF-1α expression CAIX was weakly, but statistically related in tumors with reduced oxygen-tension (pO_2) levels [68, 69]. As a result CAIX proved to be a better prognostic marker compared to HIF-1α [68]. Interestingly, it has been shown that in certain cases of advanced uterine cervical cancer, CAIX expression remained high even in the absence (or low levels) of HIF-1α expression [68]. HIF-1α expression corresponded very well to the level of pO_2 present in the tumor microenvironment as per pimonidazole binding assays, a method for measuring hypoxia in tumors [69, 70]. CAIX levels however, once initiated by hypoxic-stress and upregulation of HIF-1α, were not reduced once pO_2 levels were raised to normoxic conditions [68]. In fact, CAIX levels remained high in perinecrotic regions even after reoxygenation [69-71]. It has been proposed that this effect is a direct result of CAIX's *in vivo* half-life that has been reported between 2 to 3 days [71]. The extended half-life of CAIX in progressing tumor tissue of the cervix emphasizes its benefit as a prognostic marker over other hypoxic-stressed induced factors such as HIF-1α [69, 70]. It should be noted however that this effect of high levels of CAIX during reoxygenation of the tumor microenvironment might be visible in other cancer forming tissues. In addition tumor progression has been observed to proceed in perinecrotic regions even after reoxygenation. This effect may be linked to CAIX function suggesting that it is a key factor in the metabolic and invasive pathways that contribute to aggressive tumor behavior [69-72].

Similar trends of CAIX expression levels in cases of vulvar cancer have been observed [73]. Recent evidence suggests that CAIX expression in solid tumors of vulvar cancer is directly associated with poor outcomes in patients [73]. In addition, measurements of preoperative s-CAIX levels were linked to poor prognosis in these same patients [73].

This outcome resembles those seen in RCC patients mentioned previously [28, 61]. Despite the lack of information available for s-CAIX and characterization of its role in tumor progression, it still provides use as a prognostic indicator and potential therapeutic target in both vulvar cancer and RCC patients [28, 61, 73].

Head and neck cancer

Head and neck squamous cell carcinoma (HNSCC) is typically described as carcinomas occurring in the oral cavity, esophageal, and laryngeal tissues [19, 74-76]. Cases of HNSCC are usually attributed with hypoxic tumors often making treatment with chemotherapy or radiation therapy challenging [74]. As such CAIX expression has been largely associated with several cases of HNSCC [74]. As we have seen previously, the regulation of CAIX expression is coupled with levels of HIF-1α [74, 75]. Furthermore, in cases when observing oral-cavity squamous cell carcinomas, high CAIX/HIF-1α was associated with tumor necrosis, hypoxia, and microvessel density in tumors of patients [75]. CAIX expression levels were also found to be a useful indicator of reoccurrence predictability in oral squamous cell carcinoma patients that were surgically treated [77].

Similar results were seen in cases of esophageal cancers where high CAIX expression associated to diminished prognosis, therapeutic resistance, and more aggressive tumor states in patients [19]. It has also been shown that CAIX expression is associated with lymph node metastases indicating that CAIX-induced alterations of the tumor milieu caused by the “hypoxic phenotype” may be preserved during migration of tumor cells [19]. Interestingly, CAIX expression was positively regulated by the tyrosine kinase Her-2 indicating a potential alternative activation pathway of CAIX expression [19]. Laryngeal carcinoma, which often originates in the glottis, with rare cases arising in the supraglottis, displays the same relationship between CAIX expression and prognosis in glottis laryngeal carcinomas [76]. CAIX expression however was absent when laryngeal carcinoma is derived from the supraglottis [76]. As a result expression levels of CAIX/HIF-1α shows a similar correlation in glottis derived laryngeal carcinomas but did not correlate to prognostic factors of supraglottic laryngeal carcinoma [76]. Overall CAIX expression has been associated with poor prognosis in HNSCC patients indicating potential as a biomarker in these cancers [19, 74-76].

Brain and astrocytoma cancer

Malignant glioblastomas are often linked to increased glycolytic metabolism and often display a high degree of intratumural hypoxia [15]. This effect is directly coupled to levels of CAIX, which exhibits no expression in normal brain tissues [15]. Interestingly, glioblastomas show no association with CAIX expression level between low-grade and high-grade gliomas [15]. CAIX was also observed to express in high levels in astrocytomas, which are highly malignant tumors derived from glial cells [78]. In addition there was no observable co-expression of CAIX with EGFR, which can upregulate HIF-1α mediated gene expression independent of hypoxia in astrocytomas [78]. Instead, similar to glioblastomas, malignant astrocytomas are strictly regulated by hypoxic-induced stress [15, 78]. Alternatively, CAIX knockdown in both tumor types resulted in reduced proliferation and migratory rates in tumor cells, and overall reduced tumor size [15, 78]. In cases of medulloblastomas (MBs), and supratentorial primitive neuroectodermal tumors (PNETs), the most common and aggressive pediatric brain tumors, high levels of CAIX expression was observed in perinecotic regions [79]. Furthermore, CAIX was able to predict prognosis in patients as per multi- and univariate analysis [79]. This result is very promising as these types of pediatric tumors usually have a low survival rate resulting in a max life span of <5 years post-diagnosis [79]. Overall CAIX is shown to be a useful prognostic indicator in both adult and pediatric brain tumors [15, 78, 79]. Furthermore, the close relationship of CAIX to metastatic behavior of each brain tumor type, coupled with the minimal CAIX expression found in normal brain tissue, presents CAIX as a promising anti-cancer target [78, 79].

Gallbladder, liver and bone-marrow cancers

Urothelial carcinoma of the bladder, which encompasses roughly 90% of all bladder cancer cases, exhibits differential expression of CAIX [80]. Specifically, CAIX is widely expressed in

low-grade invasive tumors and has shown to correlate directly to patient prognosis [80]. Integration of CAIX into prognostic models has presented to be an accurate biomarker and also a strong predictor of reoccurrence in post-operative patients [80]. In general it has been shown that the most invasive tumors that exhibit CAIX expression are of regions of necrosis indicating a hypoxic tumor microenvironment [80, 81]. In addition the presence of CAIX was shown to be necessary for metastatic characteristics of these tumors [81]. Therefore a reduction in CAIX activity may correlate to a reduction in invasive tumor behavior. Furthermore, normal urothelial tissue is null of CAIX expression making it ideal as a prognostic marker [80, 81].

Recent clinical and *in vitro* studies utilizing Hep3B, Huh-7 and HepG2 cell lines show that CAIX is expressed in aggressive cases of hepatocellular carcinoma and hepatoblastoma [82, 83]. Specifically, CAIX expression was shown to be induced via introduction of hypoxic-stress and/or transforming-growth factor-β1 (TGF-β1) [83]. TGF-β1 has routinely been observed in cases of human hepatocellular carcinoma (HCC), and has also been associated with tumor progression via immune-suppression and activation of angiogenesis [84]. An interesting observation in HCC is that inhibition of hypoxia-induced CAIX enhances the effect of chemotherapeutics that target hexokinase II [83]. Also, CAIX expression was inversely related to E-cadherin levels, an important mediator of cell adhesion [83]. This suggests CAIX may function in liver cancer cell migration and invasion. These data provide evidence that CAIX inhibition may therefore be a useful combinatorial therapy against HCC and also as a prognostic indicator.

Clinical studies involving patients with epithelial bone-marrow metastases displayed slight CAIX expression, mostly related to focal membranous-points, or areas of invading tumor cells [85]. This result however, due to the low levels of observable CAIX expression, is not significant enough to conclude CAIX to be a good prognostic or therapeutic marker for epithelial bone-marrow metastases [85]. In contrast the relation of CAIX expression to malignant tissues in bone-marrow tumors does display similarities to observations of CAIX expression profiles in other aggressive tumor tissues [85]. It should also be noted that this study also evaluated CAIX expression in cases of hematological metastases and observed absolutely no CAIX expression. This indicates CAIX may not be directly involved with hematological tumor function [85].

Gastric cancer

Unlike the previously mentioned cases, CAIX exhibits differential expression pattern in types of gastric cancer. Moreover, gastric epithelium is indicative of intrinsic CAIX expression and therefore it could be postulated that expression profiles of CAIX in cancerous forms of this tissue might not follow the trends previously observed [6, 45]. In fact, when regarding levels of CAIX in small intestinal carcinoma (SIC), which is a relatively rare but often an aggressive type of carcinoma, high CAIX levels have been shown to correlate more readily with a good prognosis in patients [86]. Good prognostic factors in this case are associated with reduced areas of moderately differentiated tumor cells, and no observable lymph node metastasis [86]. This result contrasts that which has typically been observed in other carcinomas, and hence a reduced value of CAIX as a prognostic marker in cases of SIC. Alternatively, when observing epigenetic regulations of the *CA9* promoter, it has been shown that methylation of the CpG site, responsible for *CA9* activation, occurs predominantly in cases of diffuse-type gastric cancers rather than interstitial [18]. Diffuse-type gastric carcinomas usually demonstrate highly migratory and invasive qualities [18]. Interestingly, CAIX expression in both interstitial and diffuse-type carcinomas was not regulated by hypoxia. This indicates alternative mechanisms occurring in gastric mucosa that regulate CAIX levels. Insights into this thought can be deduced via a recent study correlating suPAR levels with CAIX expression [87]. suPAR is found in a plethora of cells including vascular endothelial cells, monocytes, and neutrophils, and is thought to be associated with inflammatory response elements [87, 88]. Furthermore suPAR has shown to mediate angiogenesis, adhesion, migration, and cell proliferation during inflammation, and has also been utilized as a prognostic marker of gastric cancer [88].

The relation between suPAR and CAIX expression, and suPARs association with immuno-driven cell proliferation, migration, and adhesion, may suggest CAIX is regulated by immune response elements in gastric tissues rather than hypoxic-induced stress similar to that seen in HCC [87]. However, much more research on this relationship will need to be presented to truly make this conclusion.

In summary CAIX expression patterns display a relatively common motif between solid tumors of various tissues. As such, one can postulate the modes of expression, with the exception being cases of gastric and bone-marrow cancers and slight derivations in cases of liver, kidney, and cervical cancers, this mechanism is relatively conserved [6, 7, 17-19, 45]. Furthermore, high CAIX expression levels have been determined to be associated with more aggressive tumor tissues [17-19]. Specifically, the presence of CAIX has displayed properties as a modulator of the hypoxic tumor milieu, which ultimately translates to a tumor cells ability to rapidly breakdown glucose, prompt an acidic pHe and more neutral pHi, contribute to a tumor cell adhesion, invasion, and migration, and attribute to radiation and chemotherapeutic resistance [6-9, 17-19, 45]. It was also observed that CAIX was still present even after reoxygenation of hypoxic tumor regions, which ultimately contributed to overall poor outcomes in patients [28, 62, 63]. As we can see, CAIX has become regarded as a key mediator of the tumor milieu that equates to aggressive tumor behavior [6-10, 17-19]. In the next section we will review the role of CAIX in the tumor microenvironment and highlight its contribution to the metastatic cascade.

CAIX is essential for tumor metabolism and function

pH regulation and the metastatic cascade

The acidic extracellular pH (6.0-6.8) of metastatic tumor tissues is postulated to be directly attributed to lactic acid production by way of an increase in anerobic glycolysis, and carbonic acid produced through the pentose phosphate pathway [88]. Alternatively, the intracellular pH of tumor cells remains close to physiological levels enabling for proper mediation of metabolic and signaling pathways [4, 88]. As such, this acidic pHe and neutral pHi is established via a network of transporters and pumps allowing exchange of glucose, and glycolytic bi-products of lactate, H^+ and HCO_3^- [4, 88]. H^+ and lactate produced by glycolysis is transported out of the cell by Na^+/H^+ exchangers (NHE), H^+-ATPase, the H^+-lactate co-transporter (HCLT), and monocarboxylate transporters (MCTs) [89]. In addition, CO_2 produced in the pentose phosphate pathway readily diffuses through the tumor cell membrane where it can be converted to H^+ and HCO_3^- by CAIX [14, 29, 88]. In contrast, HCO_3^- produced from CAIX interconversion of CO_2 at the extracellular surface can be imported into the tumor cell by Na^+-HCO_3^- co-transporters (NBC) and Cl^-/HCO_3^- exchangers (AE) resulting in the buffering of neutral pHi (Figure 2A) [14, 88]. This balance between high intracellular HCO_3^- and high extracellular H^+ is critical in biological functions of tumor cells wherein ± 0.1 pH units in the cytosol of the tumor cell can disrupt ATP production, protein synthesis, cell proliferation, migration and apoptosis [14]. It has been shown in CAFs that the balance between pHe and pHi is critical for tumor cells to maintain high rates of proliferation [89]. In a paradoxical sense nevertheless, acidic extracellular tumor milieus actually produce a negative effect on proliferating rates; this attribute is overcome by the presence of high HCO_3^- concentrations in the cytosol of tumor cells [89]. The same result was observed in glioblastoma cells whereas the presence of CAIX mediated both influx and efflux rates of HCO_3^- and H^+, respectively [15]. Proper mediation of this transport mechanism occurs via the juxtamembranous position of the enzyme allowing positional control of the products in CAIX catalysis and contributing to the overall acidic phenotype [15]. The pKa of CAIX at 6.49 is postulated to be a critical evolutionary trait, such that it is able to contribute to the proton/bicarbonate efflux/influx gradient in the acidic extracellular environment without losing activity [90, 24, 31]. It has been suggested that this occurs via the carboxy-rich PG domain of the enzyme acting as an internal buffer for CAIX allowing it to uphold its high catalytic efficiency in the environment in order to mirror the increased rate of glycolysis [24]. However this notion has been challenged via *in vivo* studies that show equivalent catalytic efficiency of both

full-length (containing PG and CA domains) and truncated (CA Domain only) forms of CAIX [30, 31]. In addition, CAXII, which is also a homodimeric tumor associated CA, has shown its capability of establishing an acidic tumor milieu in both breast and lung cancer cell lines [14, 90]. Interestingly CAXII, an enzyme that lacks the PG domain extension, maintains its activity in the acidic tumor microenvironment allowing for maintenance of the proton/bicarbonate efflux/influx gradient [14, 90].

Overall, the establishment of the differential pHe and pHi associated with hypoxic and aggressive tumor cells will ultimately set the stage for metastatic cascade events [88, 90]. Acidic extracellular pH results in necrosis or apoptosis of adjacent normal cells by p53- and caspase-3-dependent mechanisms [91]. This effect, coupled with tumor cells ability to up-regulate transporters and exchangers to maintain neutral pHi, provides a variable environment for tumor invasiveness while maintaining proliferative properties [88, 91]. This effect also constitutes to the secretion of lysosomal enzymes that are optimally active in more acidic pH [88]. Specifically, MMPs and cathepsins are released, which act to degrade ECM and adjacent bone matrix, to prompt the environment for cell migration and invasion [13, 88, 90]. Alternatively, the acidic pHe also contributes to focal adhesion kinase (FAK) activity, which plays a major role in cellular motility by regulating plasma membrane localization [92]. This effect is coupled with activation of proangiogenic factors such as VEGF, which leads to increased tumor cell survival and enhanced tumor proliferation rates [39, 88]. Low pHe has also shown to induce an inflammatory response thus contributing to the pathological trigger for both inflammation and pain in cancer patients [88]. In essence this occurs by way of proton sensing GPCR activity that stimulates cyclooxygenase-2 (COX-2) expression and prostaglandin-E_2 (PGE_2) production [93]. This particular response becomes enhanced further by succinate-induced activation of HIF-1α, which therefore stimulates IL-1β [34]. The final facet that represents the metastatic cascade is the overall resistance to radiation and chemotherapies [94]. Specifically, alterations in compounds permeability, as with VEGF and EGF targeted inhibitors, show a significant decrease in efficacy in colon cancer treatments [1, 2, 94]. Alternatively, the increase in CO_2 in the hypoxic niche, followed by its rapid conversion to HCO_3^- by CA activity, contributes greatly to a reduction in ionizable free-oxygen species resulting in a decreased radiotherapeutic response [1, 2, 15]. Therefore, differential pHe and pHi modulations, aided by CAIX activity, establish the adaptive environment which ultimately contributes to therapy resistant malignant tumor formation.

Cell migration, invasion, and adhesion

Hypoxia, as discussed previously, is interconnected to cancer cell migration and invasive potential [95]. In addition HIF-1α mediated CAIX expression is directly correlated to these properties, as is lysyl oxidase (LOX), an enzyme that catalyzes crosslinking of collagens or elastins [95]. Typically, cell-cell mediated adherens junctions hold cells together with glycoproteins playing a key role in the process [96]. Cadherins, which are transmembrane Ca^{2+}-dependent homophillic adhesion receptors, induce the ubiquitous adhesive interactions required for maintenance of solid tissues [96]. Of the sort is the well characterized epithelial associated E-cadherin [96, 97]. E-cadherin has been associated with suppression of epithelial tumor invasiveness and metastasis with a loss in function equating to enhancing these aforementioned properties [96, 97]. A key regulator of E-cadherin-mediated adhesion is β-catenin [97, 98]. β-catenin is activated via stimulation from α-catenin binding [98]. Loss of β-catenin activity negatively affects E-cadherin ultimately resulting in reduced cell-cell adhesion [98]. CAIX has shown to compete with α-catenin for β-catenin binding [98, 99]. This leads to the suppression of E-cadherin leading to the unstable cell-cell interactions typically seen in metastatic tumor cells [98, 99]. Alternatively, when CAIX expression was decreased, cell adhesive properties were restored [99].

MMPs and cathepsins induce cell motility by way of matrix remodeling [88, 100]. MMP and cathepsin activity leads to extracellular matrix (ECM) degradation of parenchyma encapsulating primary tumors [100]. As in glioma cells, migration and invasion induced by MMPs occurs in essentially two phases [101]. Glioma cells attach to ECM proteins via cell adhesion receptors; this is followed

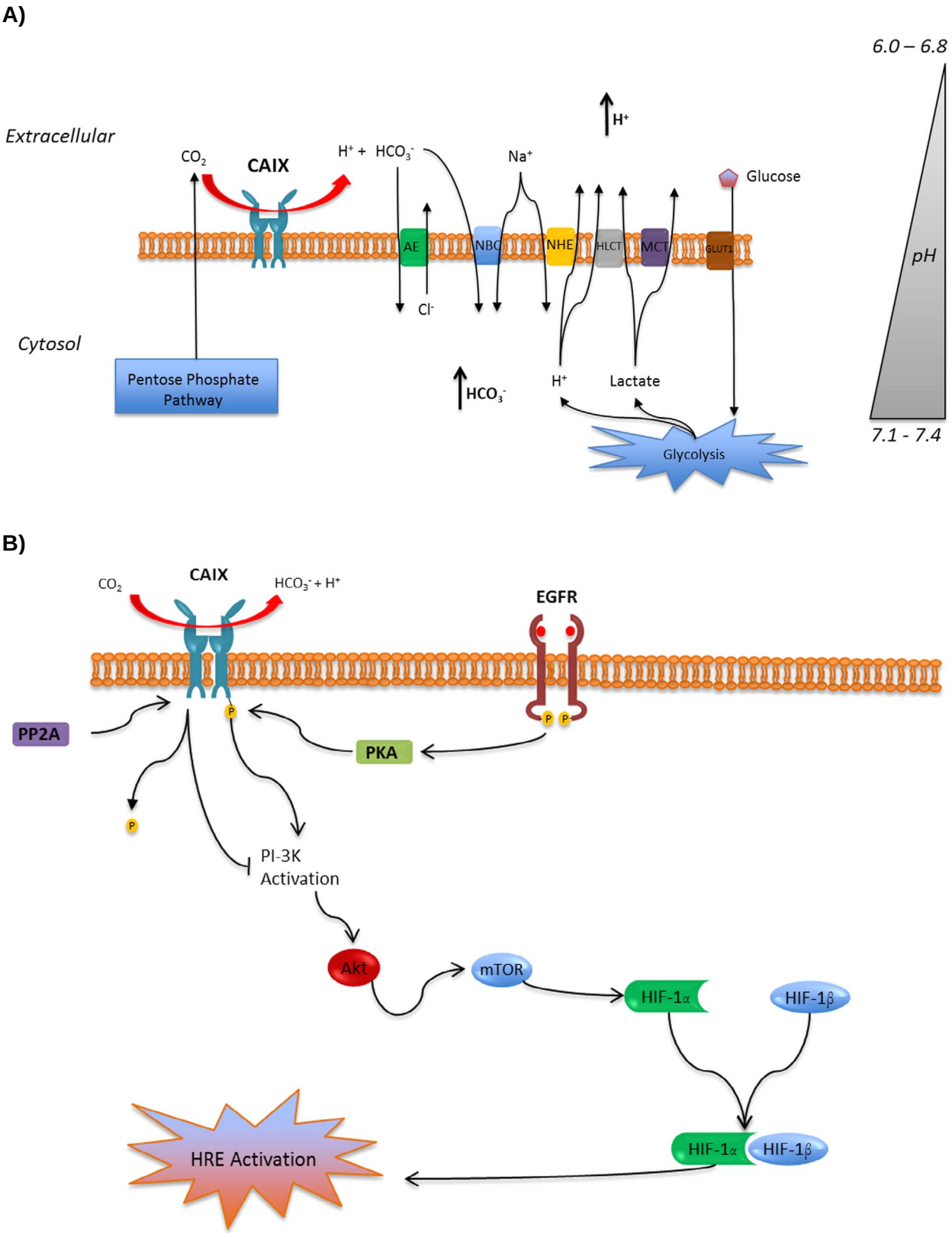
A)
6.0 – 6.8
Extracellular
CO2
CAIX
H+ + HCO3-
Na+
H+
Glucose
AE
NBC
NHE
HLCT
MCT
GLUT1
pH
Cl-
Cytosol
Pentose Phosphate Pathway
HCO3-
H+
Lactate
Glycolysis
7.1 - 7.4
B)
CO2
CAIX
HCO3- + H+
EGFR
PP2A
PKA
PI-3K Activation
Akt
mTOR
HIF-1α
HIF-1β
HRE Activation
HIF-1α
HIF-1β

Figure 2

by proteolytic degradation of the ECM by MMPs and serine proteases, and followed by adhesion protein degradation by cathepsins that result in increased cell motility [101]. All of the aforementioned proteases are secreted and activated by a reduction in extracellular pH induced by CAIX activity [101]. In contrast protease activity (and extracellular expression) is reduced upon CAIX inhibition [101]. Similarly the same effect is seen in colorectal cancer cells facilitated by stimulation of an inflammatory response; hence upregulation of COX-2 and CAIX activation induces MMP activity [102].

CAIX has been shown to hold a critical role in focal adhesion, a necessary step accompanying the migration and invasive behavior of tumor cells that leads to metastasis [103]. CAIX has been shown to co-localize at focal contacts of the ECM with paxillin, a common ECM scaffolding protein activated by FAK [104]. It has also been observed that CAIX, by utility of its PG domain, can adhere to cells via an interaction with ECM components hyaluronan and collagen [26, 104]. CAIX loses this ability upon binding of the monoclonal antibody M75, which translates into a reduction in focal adhesion abilities of tumor cells [26]. CAIX has also been proposed to play a more general role in tumor migration/adhesion/invasion phenomenon by directly interacting with the Rho/ROCK pathway that stimulates cytoskeletal rearrangement [101, 104]. It has been shown that reduced levels of CAIX correlate to suppression of Rho/ROCK pathways and FAK activation of paxiilin, both of which mediate cell motility and focal contact formation [101, 104]. Overall, the importance of CAIX in the tumor cell migration/adhesion/invasion phenomenon increases its physiological importance and thus contributes to its value as an anti-cancer target [105].

Signaling

As discussed previously, CAIX has garnered attention as not only a mediator of cell motility and pH regulation of the tumor milieu, but more recently as an important signaling molecule (Figure 2B) [17, 36]. Three phosphorylation sites have been observed within the IC domain of CAIX at Thr443, Ser448, and Tyr449, all of which are phosphorylated by protein kinase A (PKA) [34, 105]. In contrast it has been observed that de-phosphorylation of CAIX at these specific locations occurs by protein phosphatase 2 (PP2A) activity [34, 105]. Different phosphorylation combinations have been demonstrated to influence catalytic and signaling potential of CAIX [105]. It has been observed that ligand induced EGFR signaling can prompt activation of cAMP/PKA phosphorylation of Tyr449, which in turn induces activation of PI-3K signaling cascade further facilitating Akt activation [105]. This Akt activation as we have seen corresponds to an upregulation of HIF-1α in non-hypoxic conditions. This mechanism of p-CAIX mediated activation of the PI-3K pathway exhibits properties similar to a feed-forward regulator of its own expression and also other HREs that contribute to metastatic tumor cell behavior [17, 32, 36]. The other two-phosphorylation sites, Thr443 and Ser448, contribute directly to CAIX activity [105]. It has been observed that optimum CAIX activity (even more so than CA II) is achieved when Thr443 is phosphorylated by PKA. Alternatively, when Ser448 is phosphorylated

Legend to Figure 2. CAIX Functions in Tumor Microenvironment. A) The acidic extracellular pHe and near physiological intercellular pHi is established via a network of transporters and pumps in the tumor milieu. H^+ and lactate produced by glycolysis is transported out of the cell by Na^+/H^+ exchangers (NHE), H^+-ATPase (not shown), the H^+-lactate co-transporter (HCLT), and monocarboxylate transporters (MCTs). CO_2 produced in the pentose phosphate pathway readily diffuses through the tumor cell membrane where it is converted to H^+ and HCO_3^- by CAIX. The HCO_3^- produced from CAIX catalysis is imported into the tumor cell by Na^+-HCO_3^- co-transporters (NBC) and Cl^-/HCO_3^- exchangers (AE) in order to buffer the cytosol. B) EGFR signaling can induce activation of cAMP/PKA activation, which thus acts to phosphorylate CAIX at Tyr449 of the IC domain. Phosphorylated CAIX can further activate PI-3K signaling cascade leading to Akt activation. Furthermore, Akt activation corresponds to an upregulation of HIF-1α independent of O_2 concentration and thus upregulate CAIX expression. This mechanism suggests CAIX can act as a feed-forward regulator of HRE activation and thus its own expression (Figures A and B were generated based on observations seen in [4, 14, 17, 29, 32, 36, 88, 89, 105]).

catalytic activity is reduced [105]. Thus, the combination of PKA induced phosphorylation of Thr443, coupled with dephosphorylation of Ser448 by PP2A proposes a regulatory mechanism of CAIX activity in the tumor milieu [17, 105]. This phospho-regulated mechanism suggests that levels of CAIX activity are dependent on the flux of metabolic activity of the tumor cell. Additionally, this observation coupled with the fact that *in vivo* catalytic activity is not dependent on the presence of the PG domain, suggests that CAIX activity in the acidic microenvironment is most likely regulated by alterations in metabolic flux rather than buffering capabilities of the PG domain [31, 105]. Therefore, the unique attribute of the PG domain of CAIX may strictly be utilized in the enzyme's adhesion capabilities [26].

As discussed previously, CAIX also exists as the soluble s-CAIX [25, 28]. Recently, an intriguing observation has been made regarding s-CAIX in that it has potential to internalize into the cytoplasm and traffic to the nucleus [106, 28]. It has been shown that CAIX can co-localize with exportins and importins, which are important for nucleo-cytoplasmic trafficking [28]. This relationship between CAIX and importin and exportin molecules was heightened in the presence of IC domain phosphorylation at Thr443 and Tyr439 [106]. Despite this unique observation seen regarding CAIX function, trafficking of CAIX to the nucleus has so far not been directly linked to specific gene activation [106]. It can be postulated, with CAIX possessing an important role in the Rho/ROCK pathways, the FAK signaling cascade, and PI-3K/Akt activation of HIF-1α, that this internalization mechanism acts as a mediator for these pathways although this has not been observed [17, 32, 105, 106]. Furthermore, this signaling event might be a direct consequence of altered metabolic flux seen in the tumor cell such that PKA phosphorylation of Thr443 may influence this internalization. In addition dephosphorylation of this same residue may in turn reduce these signaling events resulting in a more static tumor environment. However, to fully conclude these remarks more research must be done to characterize this mechanism. In summary these observations suggest a broader physiological role of CAIX in normal tissue than previously determined [46-48].

CAIX as an anti-cancer target

As we have seen CAIX plays an important role in tumor biology, specifically in pH regulation, proliferation, cell motility, adhesion, and signaling [6, 7]. As a result CAIX has shown increased potential as not only a prognostic marker for aggressive cancers but also as an anti-cancer target [7]. In addition, CAIX has several physical characteristics that make it an optimal therapeutic target. The extracellular facing catalytic domain of CAIX eliminates drug delivery complications often exhibited by internalized or cytosolic targets [7, 23, 24]. Furthermore, intrinsic CAIX expression has shown to be limited to specific tissues reducing the potential for off-target effects seen with several anti-cancer drugs [46-48]. More importantly, knockdown or inhibition of CAIX has resulted in the decrease of both primary tumor growth and metastatic potential, and also an increase in tumor susceptibility to radiation treatment [19, 58, 66, 75, 78, 107, 108]. As a result, the interest in developing novel inhibitors of both small-molecular and biologic derivation against CAIX has arisen [6, 7]. To date, several inhibitors for CAIX have been developed. These inhibitors include sulfonamides, which are common potent CA inhibitors, coumarins, monoclonal antibodies and even peptide-based ligands (two of which are in Phase II and III clinical trials in indisulam and RECENARAX®, respectively) [6, 7, 60, 109, 110]. Despite some recent success in the development of CAIX inhibitors, the most challenging aspect has been the development of compounds with high isoform specificity. [6, 7, 111].

Utilization of structure-based drug design has been a hallmark technique to exploit the subtle differences between the active site of CAIX in comparison to other human CAs. However difficulties regarding expression and crystallization of wild-type CAIX have resulted in limited structural information as per one X-ray crystal structure (PDB: 3IAI) deposited to the Protein Data Bank (PDB) to date [24, 111]. To overcome this hindrance a pseudo-CAIX, or CAIX mimic has been developed by using the easily crystallized CA II as a template (Figure 3A) [111]. The CAIX mimic has proven useful for determining modes of binding of certain drugs, specifically sulfonamide based derivatives [111, 112]. Superposition of acetazolamide (AZM)

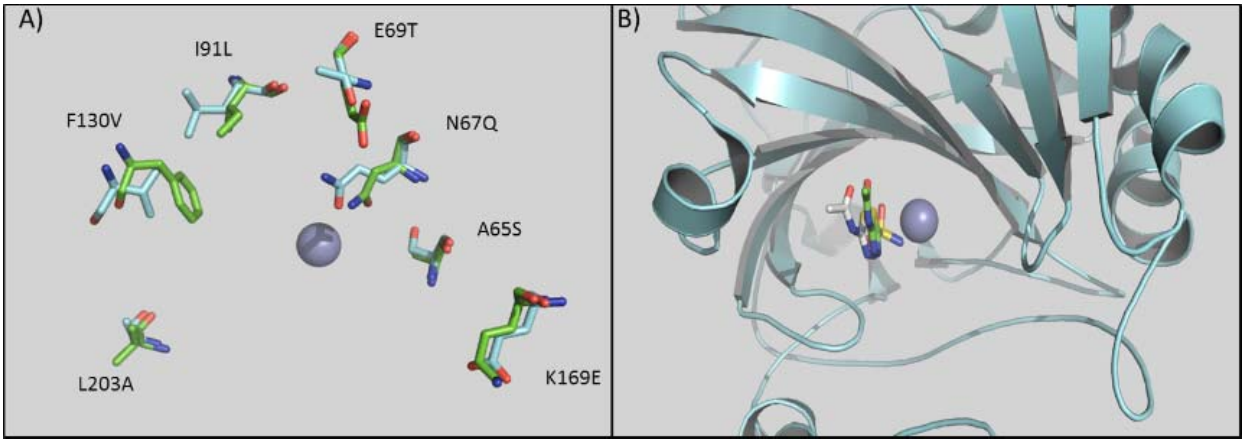

Figure 3. CAIX and CAIX mimic active site comparisons. A) Specific point mutations made in the active site of CAII (green) designed to mimic the active site of CAIX. The amino acid differences are represented as a superposition of the CAII active site residues with that of CAIX (pale cyan) (PDB 3HS4 and 3IAI, respectively), these include: A65S, N67Q, E69T, I91L, F130V, K169E, and L203A. The CAIX mimic has shown to be a valuable model for structural targeting of wild-type CAIX. B) The active site of CAIX with an overlay of acetazolamide (AZM) from 3IAI (white) and 3HS4 (green). Note that the sulfonamide group binding to the zinc is conserved, between both structures. Variations occur in the binding of the "tail-like" extension of AZM most likely due to difference in active site cleft regions located radially outward from the catalytic zinc. In addition the reduced steric hindrance from the absence of F130 (not shown) in CAIX may allow for more Van der Waals interactions in the hydrophobic cleft and might contribute to a more favorable binding of steroidal based compounds. Exploiting these slight differences between active sites of each isoform is essential when designing isoform specific inhibitors. (Figures were created in *Pymol* using coordinates from PDBs 3IAI, 3HS4, and 3DC3 from [24, 111, 112]).

from crystal structures of CA II (PDB: 3HS4), CAIX and CAIX mimic (PDB: 3DC3) shows that the sulfa-group extension binds to the zinc in a conserved manner with the amide interacting directly to the catalytic zinc (Figure 3A) [24, 113]. Alternatively, variations in binding are observed in the backbone of AZM due to the differences between CA II and CAIX hydrophobic and hydrophilic clefts [24]. This suggests that the R-groups of sulfonamide-based compounds can be manipulated to interact with small changes between the hydrophobic and hydrophilic clefts while maintaining a strong association with the active site zinc. One example of this can be seen in estradiol-derived sulfonamides, which have been designed based on previously seen anti-mitotic inhibitors [114, 115]. Structural and kinetic studies of these compounds indicate that steroidal based sulfonamide compounds are able to exploit the larger hydrophobic pocket of the CAIX active-site via an increase in Van der Waals contacts of the steroidal backbone [24, 114, 115]. The same trend was shown using energy calculations from molecular docking studies of each compound with wild-type CAIX, which also predicted similar modes of binding to that observed in the CAIX mimic [115]. Alternatively, S-glycosyl primary sulfonamides have been developed in attempts to exploit the extracellular facing catalytic domain of CAIX [116]. These particular compounds contain a hydrophilic sugar moiety, which shows limited cell permeability, adjacent to a sulfonamide group [116, 117]. In addition, the carbohydrate moiety allows for a potential increase in oral bioavailability that, when compared to current available anti-cancer therapeutics, would make delivery far less invasive [117]. More recently, notions to combine both properties of compounds with both high isoform specificity and limited membrane permeability in the form of a pro-drug have been implemented [118]. Gluco-sulfonamide based conjugates have been designed to have favorable membrane permeability and poor CA inhibitory properties until unmasked in circulation. At this point, the compounds exhibit the opposing characteristics making for a robust orally delivered

pro-drug against that specifically targets CAIX [118]. Similarly a series of carbamoylphosphonates, initially designed to inhibit MMPs, was serendipitously discovered to inhibit CAIX activity [119]. These compounds had already shown promise as non-toxic, orally administered anti-cancer therapeutics and when introduced to an acidic pH (<7.0) they become ionized and are unable to cross the cell membrane [119]. This feature resembles the properties exhibited by the aforementioned gluco-sulfonamide pro-drug approach. In this particular case drug efficacy becomes dependent on the acidic tumor milieu [118, 119]. Therefore by combining both structural and pharmacokinetic approaches it might be possible to develop CAIX specific drugs.

In addition to the development of small-molecule inhibitors of CAIX, there is also a demand to engineer biologic-based (antibody or peptides) inhibitors for CAIX. Utilization of the ability of monoclonal antibodies, such as M75 and G250, to recognize the PG domain of CAIX has shown effectiveness in disrupting the ability of CAIX to function in tumor cell adhesion and motility [26, 31]. This becomes promising as such monoclonal antibodies exhibit high binding affinity to the distinctive PG domain of CAIX thus circumventing the potential of non-specific CA targeting [94]. In addition peptide based inhibitors for CAIX have also been discovered utilizing a phage-display library [113]. However the benefit of this type of ligand is still unclear. There is postulation that the specific binding region of such peptides can further be exploited for the development of a peptide-based inhibitor that is specific for CAIX targeting [109].

CONCLUSION

Since its discovery, CAIX has gained a large amount of attention in terms of its role in tumor biology [6, 7, 17-19]. Furthermore, characterization of the physiological properties of CAIX proposes it to be an essential regulator of metabolism, cell motility, and overall metastatic behavior in solid tumors [5-7, 44]. Additionally the inhibition or knockdown of CAIX in several cancers has proven detrimental to overall tumor growth and metastatic properties [19, 58, 66, 73-75, 78, 107, 108]. Therapeutic targeting of CAIX has presented to be advantageous in terms of increasing radiation and chemosensitivity in certain cancers [120]. As such, CAIX still remains an elusive drug target bearing its high level of homology between other ubiquitously expressed isoforms of CA [5-7, 24]. However recent developments in engineering CAIX specific inhibitors have shown great promise in overcoming this challenge [5-7, 62, 109, 110]. Despite such progress however, very few inhibitors have been used in a clinical setting. Moreover, structural information for wild-type CAIX still remains limited [24]. In summary CAIX has revealed great potential as a prognostic marker and as a therapeutic target for several cancers. Thus, the development of potent CAIX specific inhibitors will enhance current anti-cancer treatment.

ACKNOWLEDGEMENTS

We would like to thank the full gamut of CA researchers that have allowed us to write this review. This work was funded in part by a National Institutes of Health grant CA165284.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest related to the content of this review.

REFERENCES

1. Moulder, J. E. and Rockwell, S. 1987, Cancer Metastasis Rev., 5, 313.
2. Peskin, B. S. and Carter, M. J. 2008, Medical Hypotheses, 70, 298.
3. Racker, E. 1981, Science, 213, 1313.
4. Warburg, O., Geissler, A. W. and Lorenz, S. 1967, Hoppe-Seyler's Z. Physiol. Chem., 348, 1686.
5. Sadri, N. and Zhang, P. 2013, Cancers, 5, 320.
6. McDonald, P. C., Winum, J.-Y., Supuran, C. T. and Dedhar, S. 2012, Oncotarget, 3, 84.
7. Neri, D. and Supuran, C. T. 2011, Nat. Rev. Drug Discov., 10, 767.
8. Aggarwal, M., Boone, C. D., Kondeti, B. and McKenna, R. 2013, J. Enzyme Inhib. Med. Chem., 28, 267.
9. Aggarwal, M., Kondeti, B. and McKenna, R. 2013, Bioorg. & Med. Chem., 21, 1526.
10. Lindskog, S. and Silverman, D. N. 2000, EXS, 175.
11. Liao, S.-Y., Lerman, M. I. and Stanbridge, E. J. 2009, BMC Developmental Biology, 9, 22.

12. Türeci, O., Sahin, U., Vollmar, E., Siemer, S., Göttert, E., Seitz, G., Parkkila, A. K., Shah, G. N., Grub, J. H., Pfreundschuh. and Sly, W. S. 1998, Proc. Natl. Acad. Sci. USA, 95, 7608.
13. Ilie, M., Hofman, V., Zangari, J., Chiche, J., Mouroux, J., Mazure, N. M., Pouysségur, J., Brest, P. and Hofman, P. 2013, Lung Cancer, 82, 16.
14. Chiche, J., Ilc, K., Laferriere, J., Trottier, E., Dayan, F., Mazure, M. N., Brahimi-Horn, M. C. and Pouysségur, J. 2009, Cancer Res., 69, 358.
15. Proescholdt, M. A., Merrill, M. J., Stoerr, E.-M., Lohmeier, A., Pohl, F. and Brawanski, A. 2012, Neuro-Oncology, 14, 1357.
16. Dorai, T., Sawczuk, I. S., Pastorek, J., Wiernik, P. H. and Dutcher, J. P. 2005, European Journal of Cancer, 41, 2935.
17. Wykoff, C. C., Beasley, N. J., Watson, P. H., Turner, K. J., Pastorek, J., Sibtain, A., Wilson, G. D., Turley, H., Talks, K. L., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J. and Harris, A. L. 2000, Cancer Res., 60, 7075.
18. Nakamura, J., Kitajima, Y., Kai, K., Hashiguchi, K., Hiraki, M., Noshiro, H. and Miyazaki, K. 2011, Am. J. Patho., 178, 515.
19. Birner, P., Jesch, B., Friedrich, J., Riegler, M., Zacherl, J., Hejna, M., Wrba, F., Schultheis, A. and Schoppmann, S. F. 2011, Annals of Surgical Oncology, 18, 3330.
20. Pastorek, J., Pastoreková, S., Callebaut, I., Mornon, J. P., Zelník, V., Opavský, R., Zat'ovicová, M., Liao, S., Portetelle, D. and Stanbridge, E. J. 1994, Oncogene, 9, 2877.
21. Opavský, R., Pastoreková, S., Zelník, V., Gibadulinová, A., Stanbridge, E. J., Závada, J., Kettmann, R. and Pastorek, J. 1996, Genomics, 33, 480.
22. Grabmaier, K., Vissers, J. L., De Weijert, M. C., Oosterwijk-Wakka, J. C., Van Bokhoven, A., Brakenhoff, R. H., Noessner, E., Mulders, P. A., Merkx, G., Figdor, C. G., Adema, G. J. and Oosterwilk, E. 2000, Int. J. Cancer, 85, 865.
23. Alterio, V., Hilvo, M., Di Fiore, A., Supuran, C. T., Pan, P., Parkkila, S., Scaloni, A., Pastorek, J., Pastorekova, S., Pedone, C., Scozzafava, A., Monti, S. M. and De Simone, G. 2009, Proc. Natl. Acad. Sci. USA, 106, 16233.
24. De Simone, G. and Supuran, C. T. 2010, Biochim. Biophys. Acta, 1804, 404.
25. Barathova, M., Takacova, M., Holotnakova, T., Gibadulinova, A., Ohradanova, A., Zatovicova, M., Hulikova, A., Kopacek, J., Parkkila, S., Supuran, C. T., Pastorekova, S. and Pastorek, J. 2008, Br. J. Cancer, 98, 129.
26. Závada, J., Závadová, Z., Pastorek, J., Biesová, Z., Jezek, J. and Velek, J. 2000, Br. J. Cancer, 82, 1808.
27. Hilvo, M., Baranauskiene, L., Salzano, A. M., Scaloni, A., Matulis, D., Innocenti, A., Scozzafava, A., Monti, S. M., Di Fiore, A., De Simone, G., Lindfors, M., Jänis, J., Valjakka, J., Pastoreková, J., Kulomaa, M. S., Nordlund, H. R., Supuran, C. T. and Parkkila, S. 2008, J. Biol. Chem., 283, 27799.
28. Závada, J., Závadová, Z., Pastorek, J., Biesová, Z., Jezek, J. and Velek, J. 2003, Br. J. Cancer, 89, 1067.
29. Wingo, T., Tu, C., Laipis, P. J. and Silverman, D. N. 2001, Biochem. Biophys. Res. Commun., 288, 666.
30. Hurt, J. D., Tu, C., Laipis, P. J. and Silverman, D. N. 1997, J. Biol. Chem., 272, 13512.
31. Buanne, P., Renzone, G., Monteleone, F., Vitale, M., Monti, S. M., Sandomenico, A., Garbi, C., Montanaro, D., Accardo, M., Troncone, G., Zatovicova, M., Csaderova, L., Supuran, C. T., Pastorekova, S., Scaloni, A., De Simone, G. and Zambrano, N. 2013, J. Proteome Res., 12, 282.
32. Dorai, T., Sawczuk, I. S., Pastorek, J., Wiernik, P. H. and Dutcher, J. P. 2005, Eur. J. Cancer, 41, 2935.
33. Li, Y., Tu, C., Wang, H., Silverman, D. N. and Frost, S. C. 2011, J. Bio. Chem., 286, 15789.
34. Sendoel, A., Kohler, I., Fellmann, C., Lowe, S. W. and Hengartner, M. O. 2010, Nature, 465, 577.
35. Carmeliet, P., Dor, Y., Herbert, J. M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., Koch, C. J., Ratcliffe, P., Moons, L., Jain, R. K., Collen, D., Keshert, E. and Keshet, E. 1998, Nature, 394, 485.

36. Agarwal, E., Brattain, M. G. and Chowdhury, S. 2013, Cell. Signal., 25, 1711.
37. Tannahill, G. M., Curtis, A. M., Adamik, J., Palsson-McDermott, E. M., McGettrick, A. F., Goel, G., Frezza, C., Bernard, N. J., Kelly, B., Foley, N. H., Zheng, L., Gardet, A., Tong, Z., Jany, S. S., Corr, S. C., Haneklaus, M., Caffrey, B. E., Pierce, K., Walmsley, S., Beasley, F. C., Cummins, E., Nizet, V., Whyte, M., Taylor, C. T., Lin, H., Masters, S. L., Gottlieb, E., Kelly, V. P., Clish, C., Auron, P. E., Xavier, R. J. and O'Neil, L. A. 2013, Nature, 496, 238.
38. Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R. and Ratcliffe, P. J. 1999, Nature, 399, 271.
39. Yasinska, I. M., Gibbs, B. F. Lall, G. S. and Sumbayev, V. V. 2013, Cell. Mol. Life Sci., 71, 699.
40. Hsu, C.-C., Wang, C.-H., Wu, L.-C., Hsia, C.-Y., Chi, C.-W., Yin, P.-H., Chang, C.-J., Sung, M.-T., Wei, Y.-H., Lu, S.-H. and Lee, H.-C. 2013, Biochim. Biophys. Acta., 1830, 4743.
41. Mukhopadhyay, D., Tsiokas, L., Zhou, X. M., Foster, D., Brugge, J. S. and Sukhatme, V. P. 1995, Nature, 375, 577.
42. Shweiki, D., Itin, A. Soffer, D. and Keshet, E. 1992, Nature, 359, 843.
43. Zhou, Z.-L., Luo, Z.-G., Yu, B., Jiang, Y., Chen, Y., Feng, J.-M., Dai, M., Tong, L.-J., Li, Z., Li, Y.-C., Ding, J. and Miao, Z.-H. 2010, Mol. Cancer, 9, 268.
44. Zepeda, A. B., Pessoa, A., Castillo, R. L., Figueroa, C. A., Pulgar, V. M. and Farías, J. G. 2013, Cell Biochem. Funct., 31, 451.
45. Takacova, M., Holotnakova, T., Vondracek, J., Machala, M., Pencikova, K., Gradin, K., Poellinger, L., Pastorek, J., Pastorekova, S. and Kopacek, J. 2009, Biochem. J., 419, 419.
46. Saarnio, J., Parkkila, S., Parkkila, A.-K., Waheed, A., Casey, M. C., Zhou, X. Y., Pastorekova, S., Pastorek, J., Karttunen, T., Haukipuro, K., Kairaluoma, M. I. and Sly, W. S. 1998, J. Histochem. Cytochem., 46, 497.
47. Saarnio, J., Parkkila, S., Parkkila, A. K., Haukipuro, K., Pastoreková, S., Pastorek, J., Kairaluoma, M. I. and Karttunen, T. J. 1998, Am. J. Pathol., 153, 279.
48. Li, L. and Neaves, W. B. 2006, Cancer Res., 66, 4553.
49. Medley, T. L., Furtado, M., Lam, N. T., Idrizi, R., Williams, D., Verma, P. J., Costa, M. and Kaye, D. M. 2013, PLoS ONE, 8, e80280.
50. Leibovich, B. C., Sheinin, Y., Lohse, C. M., Thompson, R. H., Cheville, J. C., Zavada, J. and Kwon, E. D. 2007, J. Clin. Oncol., 25, 4757.
51. Ivanov, S., Liao, S. Y., Ivanova, A., Danilkovitch-Miagkova, A., Tarasova, N., Weirich, G., Merrill, M. J., Proescholdt, M. A., Oldfield, E. H., Lee, J., Zavada, J., Waheed, A., Sly, W., Lerman, M. I. and Stanbridge, E. J. 2001, Am. J. Pathol., 158, 905.
52. Bartošová, M., Parkkila, S., Pohlodek, K., Karttunen, T. J., Galbavý, Š., Mucha, V., Harris, A. L., Pastorek, J. and Pastoreková, S. 2002. J. Pathol., 197, 314.
53. Colpaert, C. G., Vermeulen, P. B., Fox, S. B., Harris, A. L., Dirix, L. Y. and Van Marck, E. A. 2003, Breast Cancer Res. Treat., 81, 137.
54. Kim, S., Jung, W. H. and Koo, J. S. 2013, J. Breast Cancer, 16, 146.
55. Furjelová, M., Kovalská, M., Jurková, K., Horáček, J., Carbolová, T. and Adamkov, M. 2013, Acta Histochem., 116, 89.
56. Kim, S. J., Rabbani, Z. N., Vollmer, R. T., Schreiber, E.-G., Oosterwijk, E., Dewhirst, M. W., Vujaskovic, Z. and Kelley, M. J. 2004, Clin. Cancer Res., 10, 7925.
57. Kim, S. J., Rabbani, Z. N., Dewhirst, M. W., Vujaskovic, Z., Vollmer, R. T., Schreiber, E.-G., Oosterwijk, E. and Kelley, M. J. 2005, Lung Cancer, 49, 325.
58. Ashida, S., Nishimori, I., Tanimura, M., Onishi, S. and Shuin, T. 2002, J. Cancer Res. Clin. Oncol., 128, 561.
59. Pena, C., Lathia, C., Shan, M., Escudier, B. and Bukowski, R. M. 2010, Clin. Cancer Res., 16, 4853.
60. Kim, H. S., Kim, W. S., Park, S. H., Jung, C. W., Choi, H. Y., Lee, H. M., Jeon, S. S., Ha, H., Hwang, I. G., Lee, S. and Lim, H. Y. 2010, Urol. Oncol., 28, 157.

61. Siebels, M., Rohrmann, K., Oberneder, R., Stahler, M., Haseke, N., Beck, J., Hofmann, R., Kindler, M., Kloepfer, P. and Stief, C. 2010, World J. Urol., 29, 121.
62. Li, G., Feng, G., Gentil-Perret, A., Genin, C. and Tostain, J. 2008, J. Urol., 180, 510.
63. Zhang, B. Y., Thompson, R. H., Lohse, C. M., Dronca, R. S., Cheville, J. C., Kwon, E. D. and Leibovich, B. C. 2013, BJU Int., 111, 1046.
64. Furlan, D., Sahnane, N., Carnevali, I., Cerutti, R., Bertoni, F., Kwee, I., Uccella, S., Bertolini, V., Chiaravalli, A. M. and Capella, C. 2008, Hum. Pathol., 39, 1483.
65. Korkeila, E. A., Sundström, J., Pyrhönen, S. and Syrjänen, K. 2011, Anticancer Res., 31, 4529.
66. Lee-Kong, S. A., Ruby, J. A., Chessin, D. B., Pucciarelli, S., Shia, J., Riedel, E. R., Nitti, D. and Guillem, J. G. 2012, Dis. Colon Rectum, 55, 990.
67. Carlin, S., Khan, N., Ku, T., Longo, V. A., Larson, S. M. and Smith-Jones, P. M. 2010, PLoS ONE, 5, e10857.
68. Kirkpatrick, J. P., Rabbani, Z. N., Bentley, R. C., Hardee, M. E., Karol, S., Meyer, J., Oosterwijk, E., Havrilesky, L., Secord, A. A., Vujaskovic, Z., Dewhirst, M. W. and Jones, E. L. 2008, Biomark Insights, 3, 45.
69. Hutchison, G. J., Valentine, H. R., Loncaster, J. A., Davidson, S. E., Hunter, R. D., Roberts, S. A., Harris, A. L., Stratford, I. J., Price, P. M. and West, C. M. L. 2004, Clin. Cancer Res., 10, 8405.
70. Jankovic, B., Aquino-Parsons, C., Raleigh, J. A., Stanbridge, E. J., Durand, R. E., Banath, J. P., MacPhail, S. H. and Olive, P. L. 2006, Cytometry B. Clin. Cytom., 70B, 45.
71. Scigliano, S., Pinel, S., Poussier, S., Fouyssac, F., Plenat, F., Karcher, G. and Chastagner, P. 2008, Int. J. Oncol., 32, 69.
72. Sobhanifar, S., Aquino-Parsons, C., Stanbridge, E. J. and Olive. P. 2005, Cancer Res., 65, 7259.
73. Kock, L., Mahner, S., Choschzick, M., Eulenburg, C., Milde-Langosch, K., Schwarz, J., Jaenicke, F., Müller, V. and Woelber, L. 2011, Int. J. Gynecol. Cancer, 21, 141.
74. Beasley, N. J., Wykoff, C. C., Watson, P. H., Leek, R., Turley, H., Gatter, K., Pastorek, J., Cox, G. J., Ratcliffe, P. and Harris, A. L. 2001, Cancer Res., 61, 5262.
75. Eckert, A. W., Lautner, M. H. W., Schütze, A., Bolte, K., Bache, M., Kappler, M., Schubert, J., Taubert, H. and Bilkenroth, U. 2009, J. Oral Pathol. Med., 39, 313.
76. Wachters, J. E., Schrijvers, M. L., Slagter-Menkema, L., Mastik, M., de Bock, G. H., Langendijk, J. A., Kluin, P. M., Schuuring, E., van der Laan, B. F. A. M. and van der Wal, J. E. 2013, Laryngoscope, 123, 2154.
77. Choi, S.-W., Kim, J.-Y., Park, J.-Y., Cha, I.-H., Kim, J. and Lee, S. 2008, Hum. Pathol., 39, 1317.
78. Haapasalo, J. A. 2006, Clin. Cancer Res., 12, 473.
79. Nordfors, K., Haapasalo, J., Korja, M., Niemelä, A., Laine, J., Parkkila, A.-K., Pastorekova, S., Pastorek, J., Waheed, A., Sly, W. S., Parkkila, S. and Haapasalo, H. 2010, BMC Cancer, 10, 148.
80. Klatte, T., Seligson, D. B., Rao, J. Y., Yu, H., de Martino, M., Kawaoka, K., Wong, S. G., Belldegrun, A. S. and Pantuck, A. J. 2009, Cancer, 115, 1448.
81. Eustace, A., Irlam, J. J., Taylor, J., Denley, H., Agrawal, S., Choudhury, A., Ryder, D., Ord, J. J., Harris, A. L., Rojas, A. M., Hoskin, P. J. and West, C. M. L. 2013, Radiother. and Oncol., 108, 40.
82. Yıldırım, H., and Köçkar, F. 2009, Cell Bio. Int., 33, 1002.
83. Yu, S., Yoon, J., Lee, J., Myung, S., Jang, E., Kwak, M., Cho, E., Jang, J., Kim, Y. and Lee, H. 2011, Acta Pharmacol. Sin., 32, 912.
84. Ito, N., Kawata, S., Tamura, S., Shirai, Y., Kiso, S., Tsushima, H. and Matsuzawa, Y. 1995. Cancer Lett., 89, 45.
85. De Raeve, H. R., Vermeulen, P. B., Vanderkerken, K., Harris, A. L. and Van Marck, E. 2003, Virchows Arch., 1, 27.
86. Gu, M. J. and Kwon. K. W. 2013, J. Histochem. Cytochem., 62, 205.
87. Eugen-Olsen, J., Andersen, O., Linneberg, A., Ladelund, S., Hansen, T. W., Langkilde, A., Petersen, J., Pielak, T., Møller, L. N., Jeppesen, J., Lyngbaek, S., Fenger, M., Olsen, M. H., Hildebrandt, P. R., Borch-Johnsen, K., Jørgensen, T. and Haugaard, S. B. 2010, J. Intern. Med., 268, 296.

88. Kato, Y., Ozawa, S., Miyamoto, C., Maehata, Y., Suzuki, A., Maeda, T. and Baba, Y. 2013, Cancer Cell Int., 13, 89.
89. Santi, A., Caselli, A., Paoli, P., Corti, D., Camici, G., Pieraccini, G., Taddei, M. L., Serni, S., Chiarugi, P. and Cirri, P. 2013, Cell Commun. Signal., 11, 81.
90. Li, Y., Wang, H., Oosterwijk, E., Selman, Y., Mira, J. C., Medrano, T., Shiverick, K. T. and Frost, S. C. 2009, Cancer Invest., 27, 613.
91. Klein, C. A. 2008, Science, 321, 1785.
92. Gatenby, R. A. and Gillies, R. J. 2004. Nat. Rev. Cancer, 4, 891.
93. Ilic, D., Mao-Qiang, M., Crumrine, D., Dolganov, G., Larocque, N., Xu, P., Demerjian, M., Brown, B. E., Lim, S.-T., Ossovskaya, V., Schlaepfer, D. D., Fisher, S. J., Feingold, K. R., Elias, P. M. and Mauro, T. M. 2007, Am. J. Pathol., 170, 2055.
94. Tomura, H., Wang, J.-Q., Liu, J.-P., Komachi, M., Damirin, A., Mogi, C., Tobo, M., Nochi, H., Tamoto, K., Im, D.-S., Sato, K. and Okajima, F. 2008, J. Bone Miner. Res., 23, 1129.
95. Mao, Q., Zhang, Y., Fu, X., Xue, J., Guo, W., Meng, M., Zhou, Z., Mo, X. and Lu, Y. 2012, J. Cancer Res. Clin. Oncol., 139, 211.
96. Gumbiner, B. M. 1996, Cell, 84, 345.
97. Guilford, P. 1999, Mol. Med. Today, 5, 172.
98. Švastová, E., Žilka, N., Zat'ovičová, M., Gibadulinová, A., Čiampor, F., Pastorek, J. and Pastoreková, S. 2003, Exp. Cell Res., 290, 332.
99. Shin, H.-J., Rho, S. B., Jung, D. C., Han, I.-O., Oh, E.-S. and Kim, J.-Y. 2011, J. Cell Science, 124, 1077.
100. Robey, I. F. and Nesbit, L. A. 2013, BioMed Res. Int., 2013, 1.
101. Zhuang, T., Chelluboina, B., Ponnala, S., Velpula, K. K., Rehman, A. A., Chetty, C., Zakharian, E., Rao, J. S. and Veeravalli, K. K. 2013, BMC Cancer, 13, 590.
102. Sansone, P., Piazzi, G., Paterini, P., Strillacci, A., Ceccarelli, C., Minni, F., Biasco, G., Chieco, P. and Bonafè, M. 2009, J. Cell. Mol. Med., 13, 3876.
103. Csaderova, L., Debreova, M., Radvak, P., Stano, M., Vrestiakova, M., Kopacek, J., Pastorekova, S. and Svastova, E. 2013, Front. Physiol., 4, 271.
104. Kopacek, J. 2013, Oncol. Rep., 29, 1147.
105. Ditte, P., Dequiedt, F., Svastova, E., Hulikova, A., Ohradanova-Repic, A., Zatovicova, M., Csaderova, L., Kopacek, J., Supuran, C. T., Pastorekova, S. and Pastorek, J. 2011, Cancer Res., 71, 7558.
106. Zatovicova, M., Jelenska, L., Hulikova, A., Csaderova, L., Ditte, Z., Ditte, P., Goliasova, T., Pastorek, J. and Pastorekova, S. 2010, Curr. Pharm. Des., 16, 3255.
107. Lock, F. E., McDonald, P. C., Lou, Y., Serrano, I., Chafe, S. C., Ostlund, C., Aparicio, S., Winum, J.-Y., Supuran, C. T. and Dedhar, S. 2012, Oncogene, 32, 5210.
108. Doyen, J., Parks, S. K., Marcié, S., Pouysségur, J. and Chiche. J. 2013, Front. Oncol., 2, 199.
109. Askoxylakis, V., Garcia-Boy, R., Rana, S., Krämer, S., Hebling, U., Mier, W., Altmann, A., Markert, A., Debus, J. and Haberkorn, U. 2010, PLoS ONE, 5, e15962.
110. Dittrich, C., Zandvliet, A. S., Gneist, M., Huitema, A. D. R., King, A. A. J. and Wanders, J. 2007, Br. J. Cancer, 96, 559.
111. Genis, C., Sippel, K. H., Case, N., Cao, W., Avvaru, B. S., Tartaglia, L. J., Govindasamy, L., Tu, C., Agbandje-McKenna, M., Silverman, D. N., Rosser, C. J. and McKenna, R. 2009, Biochemistry, 48, 1322.
112. Pinard, M. A., Boone, C. D., Rife, B. D., Supuran, C. T. and McKenna. R. 2013, Bioorg. Med. Chem., 21, 7210.
113. Sippel, K. H., Robbins, A. H., Domsic, J., Genis, C., Agbandje-McKenna, M. and McKenna, R. 2009, Acta Crystallogr. Sect. F. Struct. Biol. Cryst. Commun., 65, 992.
114. Sippel, K. H., Stander, A., Tu, C., Venkatakrishnan, B., Robbins, A. H., Abandje-McKenna, M., Joubert, F., Joubert, A. M. and McKenna, R. 2011, Lett. Drug. Discov., 8, 678.
115. Stander, B. A., Joubert, F., Tu, C., Sippel, K. H., McKenna, R. and Joubert, A. M. 2012, Plos ONE, 7, e52205.
116. Lopez, M., Paul, B., Hofmann, A., Morizzi, J., Wu, Q. K., Charman, S. A., Innocenti, A., Vullo, D., Supuran, C. T. and Poulsen, S.-A. 2009, J. Med. Chem., 52, 6421.

117. Lopez, M., Trajkovic, J., Bornaghi, L. F., Innocenti, A., Vullo, D., Supuran, C. T. and Poulsen, S.-A. 2011, J. Med. Chem., 54, 1481.
118. Carroux, C. J., Rankin, G. M., Moeker, J., Bornaghi, L. F., Katneni, K., Morizzi, J., Charman, S. A., Vullo, D., Supuran, C. T. and Poulsen, S.-A. 2013, J. Med. Chem., 56, 9623.
119. Reich, R., Hoffman, A., Veerendhar, A., Maresca, A., Innocenti, A., Supuran, C. T. and Breuer, E. 2012. J. Med. Chem., 55, 7875.
120. Dubois, L., Peeters, S., Lieuwes, N. G., Geusens, N., Thiry, A., Wigfield, S., Carta, F., Mcintyre, A., Scozzafava, A., Dogné, J.-M., Supuran, C. T., Harris, A. L., Masereel, B. and Lambin, P. 2011, Radiother. Oncol., 99, 424.