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Chapter 13: Carbonic Anhydrase IX as an Imaging and Therapeutic Target for Tumors and Metastases

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Abstract

Carbonic anhydrase IX (CAIX) which is a zinc containing metalloprotein, efficiently catalyzes the reversible hydration of carbon dioxide. It is constitutively up-regulated in several cancer types and has an important role in tumor progression, acidification and metastasis. High expression of CAIX generally correlates with poor prognosis and is related to a decrease in the disease-free interval following successful therapy. Therefore, it is considered as a prognostic indicator in oncology.

In this review, we describe CAIX regulation and its role in tumor hypoxia, acidification and metastasis. In addition, the molecular imaging of CAIX and its potential for use in cancer detection, diagnosis, staging, and for use in following therapy response is discussed. Both antibodies and small molecular weight compounds have been used for targeted imaging of CAIX expression. The use of CAIX expression as an attractive and promising candidate marker for systemic anticancer therapy is also discussed.

1. Introduction

Carbonic anhydrases (CAs) are metalloproteins, usually containing Zinc (EC 4.2.1.1) that catalyze the reversible hydration of carbon dioxide. These are widespread in nature, being found in bacteria, plants and mammals (1, 2). Sixteen CA isoforms have been characterized to date in mammals, which differ in their cellular localization and catalytic activity, susceptibility to different inhibitors, and tissue-specific distribution. Some isoforms are strictly cytosolic: (CAI, CAII, CAIII, CAVII and CAXIII), some are membrane-associated (CAIV, CAIX, CAXII, CAXIV and CAXV), some are mitochondrial (CAVA and CAVB), and one (CAVI) is secreted into saliva and milk. There are also three non-catalytic enzymes (CAVIII, CAX and CAXI), which are named as carbonic anhydrase related proteins, CARPs. Although structurally homologous, the inactivity of these CAs is due to the absence of one or more Zn-binding histidine residues (1, 3–6). The structure and function of CA isozymes have been extensively reviewed by Hassan et al., 2012 (7).

CAs catalyze the reversible dehydration of bicarbonate ($CO_2 + H_2O \leftrightarrow HCO_3^- + H+$) (1, 2, 8); Since CO_2 is the main byproduct of all oxidative processes, the CAs play a fundamental role in maintaining acid-base equilibrium (1, 2, 9), and are involved in crucial physiological processes such as respiration, electrolyte balance, biosynthetic reactions that require bicarbonate as a substrate (gluconeogenesis, lipogenesis, and ureagenesis), bone resorption, and calcification (1, 2). Moreover, CAs are involved in pathological processes such as cancer progression, obesity and epilepsy and thus have been proposed as therapeutic targets.

A cDNA coding for the hCAIX protein was first cloned and investigated by Pastorek et al. (10), and the *CAIX* gene was further characterized by the same group (11). Isoform CAIX, originally named the “MN protein”, has been identified using a monoclonal antibody (M75) as a plasma membrane antigen detected in a human cervical carcinoma cell line, HeLa (12). The expression of MN antigen also was detected in various tumor cell lines and surgical tumor specimens, but not in the corresponding normal tissues, suggesting its potential usefulness as a tumor marker (13). Because it was the ninth mammalian CA identified, the MN protein was renamed CAIX. A renal cell cancer-associated antigen (G250) detected by mAb was then reported to be identical with MN/CAIX by another research group (14, 15).

CAIX is among the most active CAs for the CO_2 hydration reaction, and contains four domains on the basis of sequence similarity (11, 16): an N-terminal proteoglycan-like (PG) domain, a CA catalytic domain, a transmembrane segment (TM), and an intracytoplasmic (IC) portion. Other CAs, such as the cytosolic CAII and the membrane-associated CAXII lack the PG-like domain (Figure 1). It is worth mentioning that the M75 mAb binds to the repetitive epitope in the PG region, allowing selective detection of CAIX without cross-reactivity with other CAs (17). The PG-like domain immediately adjacent to the catalytic domain allows the enzyme to act efficiently at acidic pH values (18, 19). It has also been proposed that the PG domain is involved to cell adhesion and tumor invasion (17, 20, 21) via its interaction with β-catenin, which leads to a reduction in E-cadherin mediated cell-cell adhesion, thus promoting cell motility and invasion (20). In contrast to the PG domain, the CAIX catalytic domain has significant sequence identity (from 30% to 40%) to catalytic domains of other hCA isozymes. Both the CA and PG domains are glycosylated (16). The cytoplasmic tail of CAIX contains three potential phosphorylation sites (T443, S448 and Y449) that can participate in signal transduction. When tyrosine 449 of CAIX is phosphorylated, it can interact with the regulatory subunit of PI3K, which can transactivate the Ser/Thr protein kinase Akt, and have a direct impact on cellular glucose metabolism (22).

Acetazolamide is a clinically used sulfonamide-based CA inhibitor. An X-ray crystal structure of the catalytic domain of CAIX in complex with acetazolamide has been determined (Figure 1). The crystal structure has identified significant differences between CAIX and the other α-CA isoforms that also have solved three-dimensional structures. These developments have led to increased interest in the rational drug design of CAIX isozyme-specific CA inhibitors with potential for use as anticancer drugs for systemic therapy targeting disseminated disease (23). The CAIX protein assembles as a dimer which is stabilized by the formation of an intermolecular disulfide bond between the same Cys residue located on two CA catalytic domains (16, 23). The active site clefts and the PG

domains are thus located on one face of the dimer, while the C-termini are situated on the opposite face, facilitating protein anchoring to the cell membrane (23, 24). It has been shown that neither lipid raft localization nor phosphorylation is likely required for CAIX dimerization or activity in MDA-mb-231 breast cancer cell (25).

CAIX expression is restricted to a few tissues including the epithelium of the stomach (gastric mucosa), biliary tree, crypt cells of the duodenum, and the epithelium of the small intestine (26, 27), but is overexpressed in response to tumor hypoxia in many tumor types and plays a critical role in hypoxia associated tumor acidosis and development of the metastatic phenotype. The comparison of CAIX cDNA sequences from cancer cell lines compared to normal cells does not reveal mutations or alternative splicing that would indicate a different function in cancer (27). CAIX expression is associated with a poor prognosis [reviewed by (28)] and high CAIX expression is found in lymph node and distal metastases for a broad range of cancer types [see the “CAIX expression in cancer” section below]. Hence, CAIX is being pursued as a marker for development of targeted systemic therapies and diagnostic imaging probes.

2. CAIX regulation

Expression of CAIX protein is primarily regulated at the level of transcription. The promoter of the *CA9* gene contains an HRE (hypoxia responsive element) that is located immediately upstream of the transcription start site at position -3/-10. HIF-1α binds to the HRE and induces transcription of the *CA9* gene in response to hypoxia and increased cell density (29). The transcriptional regulation of CAIX has been reviewed in detail recently (30). Alternative splicing of the *CA9* transcript has been observed (31). Besides the predominant, full-length *CA9* mRNA (1.5 kb mRNA), an alternatively spliced variant, lacking exons 8/9 among 11 exons, has been detected by RT-PCR. This mRNA variant is constitutively expressed at very low levels and codes for a truncated, cytoplasmic/secreted form of CAIX without enzymatic activity. This is very important for studies related to hypoxia-related expression of *CAIX*, where the presence of the alternatively spliced variant is hypoxia-independent and can provide a false-positive signal (31). Hence, primers for qRT-PCR should be carefully designed to detect the longer transcript containing exons 8/9. The physiological role of this variant is unknown. Finally, the CAIX protein can be post-translationally modified by metalloprotease mediated shedding of the ectodomain (32).

Under normoxic conditions (Figure 2, right), HIF-1α is constitutively expressed but is rapidly degraded by the ubiquitin-28S proteasome pathway (33). Specifically, two proline residues of HIF-1α (P402 and P564) and asparagine (N803) are hydroxylated by oxygen-dependent proline hydroxylases (PHD) and factor inhibiting HIF-1 (FIH-1), respectively. PHD and FIH-1 belong to the Fe(II) and 2-oxoglutarate dioxygenase superfamily. Hydroxylated HIF-1α proteins bind to the E3 ubiquitin ligase, VHL, complex, leading to its rapid degradation by the proteasome (34). Hydroxylation of N803 by FIH further inactivates HIF-1α by inhibiting its interaction with transcriptional co-activator, CBP/p300 (34).

Under hypoxic conditions (Figure 2, left), lack of O_2 prevents the hydroxylation of HIF-1α by PHD and FIH (and thus degradation). Therefore, there is no VHL binding and HIF-1α is

stabilized. Non-hydroxylated N803 of HIF-1α allows binding of CBP/p300 to HIF-1α and eventual translocation to the nucleus for heterodimerization with HIF-1β, producing the master transcription factor, HIF-1. Binding of HIF-1β to HIF-1α is enhanced by MAPK phosphorylation of HIF-1α (35, 36). HIF-1 then binds to target genes that contain HRE sites, including, genes broadly related to iron metabolism (e.g. erythropoietin), glucose metabolism (e.g. GLUT-1 and GLUT-3), angiogenesis (e.g. vascular endothelial growth factor), and, last but not least, genes that are involved in pH regulation (e.g. CAIX and the sodium-hydrogen exchanger, NHE-1) and induces their transcription. Additional hypoxia-induced genes are involved in cell proliferation and viability, regulation of vascular remodeling and plasticity, cell adhesion, cell matrix metabolism, and other cellular processes (29, 35–37). For a detailed list of target genes, see Table 2 of Ke and Costa (38).

HIF-1α stabilization is a common observation in advanced stage invasive cancer and metastases (39) potentially by a number of mechanisms, resulting in the constitutive expression of cancer related genes, including *CA9*. One cancer related mechanism involves HIF-1α stabilization by glycolytic end-products, lactate and pyruvate (40), thus establishing the basis for a positive feedback loop for glycolysis and the Warburg Effect under hypoxic conditions. The tumor suppressor p53 is known to signal HIF-1α degradation as a response to DNA damage, down regulating CAIX expression (41). In most clear cell renal cell carcinomas (RCCs), the von Hippel-Lindau (pVHL) tumor suppressor is non-functional by mutation, hypermethylation or deletion. Non-functional VHL is unable to polyubiquitate HIF-1α and thus, HIF-1α is no longer recognized by the proteasomal degradation system. This leads to constitutive activation of HIF-1 even in normoxic conditions and, as a consequence, strong and uniform upregulation of CAIX and other HIF-1-induced genes (29, 42–44).

Recent studies have suggested that cancer stem cells (CSCs) preferentially survive in the hypoxic niche (45–47). CSCs have the ability to generate tumors and undergo the epithelial–mesenchymal transition (EMT) and are implicated in metastasis. Interestingly, a recent study showed that inhibition of CAIX activity with small-molecule inhibitors resulted in the inhibition of breast CSC expansion, leading to diminished tumor growth and metastasis (48). This result suggests that CAIX is an important therapeutic target for selectively depleting breast CSCs. The same study has identified the mTORC1 axis as a critical pathway downstream of CAIX in the regulation of CSC function and suggested that CAIX expression regulates CSC EMT by regulation of ‘stemness’ genes, such as Snail and Notch (48).

Our unpublished data and other studies have shown that expression levels of several hypoxia related proteins, including CAIX, are elevated at the invasive front of solid tumors at similar levels observed in central, perinecrotic areas (49). This invasive front is also the observed location of CSC migration (Figure 3) (50).

2.1 Cross talk between the CAs

Knockout (K/O) of *CA9* in transgenic animals resulted in homozygous animals that developed gastric hyperplasia, but no changes were observed in gastric pH, acid secretion or serum gastrin levels (51). Also no tumorogenesis was observed in *CA9* K/O mice, although aged *CA9* null mice may suffer degenerative brain disease (52). The knockout of *CA9* in

mice interestingly led to the up-regulation of CAII, while expression of CAIX is upregulated about 2 fold in *CA2* null mice (53). Chiche and his colleagues also observed that knockdown of *CA9* in LS174Tr colon carcinoma cells led to partial compensation by up-regulation of CAXII *in vitro* and *in vivo* (54), suggesting cross talk between CAs and that such cross talk is needed for cell pH homeostasis (51, 52). The mechanism of this specific cross talk between the CAs remains unclear (55).

3. CAIX expression in tumor hypoxia

Hypoxia can be defined as the lack of oxygen supply in a tissue such that cellular mechanisms are compromised or an adaptive response occurs. Solid tumors usually have heterogenous regions of hypoxia due to insufficient supply of oxygen by irregular and functionally defective tumor vasculature. This situation creates selective pressure in favor of tumor cells that can adapt to this microenvironmental stress, with oncogenic alterations in metabolism as a result. These adaptive changes also result in expansion of cells with more aggressive phenotypes and increased metastatic potential (56).

The importance of hypoxia has been demonstrated clinically, where it is an independent predictor of poor survival in several types of cancer. In fact, hypoxia is correlated with malignant progression and resistance to conventional chemo- and radio-therapies in many cancer types (57). This resistance is partially due to poor perfusion, which limits drug delivery and tumor penetration. Also, since chemotherapeutic agents target rapidly dividing cells, often they fail to target cells in hypoxic areas which are more slowly growing or non-proliferating. Additionally, upregulation of HIF-1 can lead to chemoresistance in tumors and metastases (e.g. HIF-1 is able to activate the multidrug resistance 1 (MDR1) gene in response to hypoxia) (58, 59). Finally, radiotherapy-based treatments which rely on the generation of oxygen super-radicals also often fail to provide effective treatment because of inadequate oxygen within the tumor mass (60).

These properties of tumor hypoxia make relevant the ability to detect hypoxic regions in order to determine which patients could benefit from adjunctive anti-hypoxia therapy and to monitor hypoxia targeted therapy response (61). Consequently, significant effort has been spent to qualify reliable hypoxia biomarkers for both diagnostic and therapeutic purposes. An exciting advance in this field has been the recent development of hypoxia-activated pro-drugs (62), which specifically target the hypoxic niche with high dose systemic chemotherapy.

Although different methods such as direct tumor pO_2 measurement by microelectrode (63), PET probes based on bioreductive 2-nitroimidazoles following intravenous administration (^{18}F-misonidazole, ^{18}F-HX4, ^{18}F-FAZA and ^{18}F-EF5 [reviewed by (64)]) have been used to detect hypoxia, each of these methods have some disadvantages. For example microelectrode use is constrained by the difficulty of accessing tumors, cost, invasiveness, inter-observer variability (65), failure to distinguish necrosis from hypoxia (66) and difficulty in calibration (61). Bioreductive-based probes can be limited by poor tumor-to-background ratios and non-specific signal generated by oxygen dependent metabolism of these probes (67).

As mentioned above, at the molecular level, hypoxia inducible factor 1 (HIF-1) is up-regulated in response to oxygen deprivation, binds to the hypoxia responsive element (HRE), and controls the transcriptional activity of numerous downstream genes, such as CAIX, which has been emerged as one of the most promising endogenous markers of cellular hypoxia.

Notably, however, staining of CAIX is not in complete agreement with pimonidazole (a hypoxia marker) staining in patient tumors and human xenograft tumors in mice (68–72). This difference may arise from short-lived fluctuations in tumor perfusion and slow rates of CAIX degradation (73), or that the pimonidazole threshold for labeling is <1.3% oxygen, whereas up-regulation of molecular markers such as CAIX occur at pO_2 values over a larger range, 0.2–2% oxygen (74).

Also, although CAIX expression correlates with the pattern of HIF-1α expression in most regions (54), exceptions have been observed where expression of CAIX is detected in the absence of HIF-1α staining (75), and these mismatches may be informative. These observations could be due to the different half-lives of HIF-1α and CAIX expression where CAIX protein is more stable (76, 77), i.e. HIF-1α is rapidly degraded in normoxic conditions and just as rapidly stabilized in hypoxic conditions. Therefore, cells that had been hypoxic and then were reoxygenated may stain for CAIX but not HIF-1α (30). In addition, when cells or tissue have only recently become hypoxic, they may stain positive for HIF-1α but not CAIX, because those cells may have been analyzed before the full onset of CAIX expression (30). Also, the duration and severity of hypoxia identified as acute, cycling (acute hypoxia followed by rapid reoxygenation), and chronic hypoxia can have differential effects on transcription and translation of HIF clients (78–80). The use of CAIX as a biomarker for imaging of hypoxia is discussed in the following section.

4. The role of CAIX in metabolism and metastasis: Acid mediated invasion

Tumor acidosis, low tumor pH, is a commonly observed consequence of dysangiogenesis and increased metabolism, which are ‘hallmarks’ of cancer. Acidosis results from enhanced rates of glucose fermentation (conversion to lactic acid) and the inefficient removal of acidic end products because of deficient vasculature in the tumor mass (81–83). Tumor acidosis has been reported to favor metastasis (84). Therefore it is considered an attractive therapeutic target.

Since intracellular (pHi) regulation controls multiple cellular functions such as energy production, proliferation and migration, tumor cells must develop strategies to regulate their pHi to survive and proliferate (85–87). In fact, tumor cells have highly sophisticated mechanisms involving a variety of proteins and buffering systems that lead to maintenance of a moderately alkaline pHi in tumor cells while generating a markedly acidic extracellular environment, allowing them not only to survive and proliferate in hypoxia, but also favoring metastasis [reviewed by (88, 89)].

As described above, HIF-1 is activated under hypoxic conditions and in many cases it is constitutively activated in tumor cells. HIF-1 acts to regulate pH homeostasis by up-regulating expression of membrane located transporters, exchangers, pumps and ecto-

enzymes such as Na^+/H^+ exchanger (NHE-1), H+-ATPases and monocarboxylate transporters (MCTs), that actively export acids from tumor cells, Cl^-/HCO_3^- exchangers (AE) that make the cytoplasm alkaline by pumping the weak base HCO_3^- into the cells, and carbonic anhydrases (CAs) (83, 90–93).

CAs alone can contribute to pH regulation, i.e. raising intracellular pH and lowering extracellular pH. Intracellular CAs can mediate the conversion of intracellular HCO_3- (a product of AE) into CO_2. This process consumes an intracellular proton. The resulting CO_2 then diffuses through the plasma membrane possibly through an aquaporin channel, wherein it is reconverted back into bicarbonate and protons by the extracellular carbonic anhydrases, e.g. CAIX, particularly in the context of a hypoxic microenvironment (94). Thus, the net effect is the movement of a proton from the cytosol to the extracellular space. The produced protons contribute to extra-cellular acidosis, leading to aggressive tumor phenotype by promoting invasion and metastasis (95). The newly generated HCO_3^- ions can then be transported back into the tumor cells by HCO_3^- transport proteins. The coupled transport process is probably essential for hypoxic cancer cells to buffer and maintain intracellular pH at near neutral conditions which is necessary for their biosynthetic reactions (26, 30, 54, 93, 94, 96). It has been shown that knockdown of CAIX minimizes the gradient in extracellular pH that occurs across three dimensional tumor spheroids, which normally have an acidic core, with a corresponding decrease in the intracellular pH of hypoxic tumor cells (97).

As mentioned previously, the crystal structure of the CAIX catalytic domain has confirmed the dimeric nature of the enzyme and showed that the N-terminal regions of both monomers are located on the same face of the dimer, while both C-termini are located on the opposite face (23). This structural organization allows for concomitant orientation of both PG domains toward the extracellular environment at the entrance to the active site clefts. This positioning of the PG domain at the border of the active site suggests a role in assisting the CA domain-mediated catalysis. In fact, the influence of the PG domain on the pH-dependent profile of the k_{cat}/K_M values for CO_2 hydration was demonstrated in a recent study (23); while the CAIX catalytic domain alone presented an optimal pK_a at 7.01, this value changed to 6.49 in the presence of the PG domain. Therefore, the PG domain of CAIX enables the hydration of CO_2 to occur at the low pH levels observed in the tumor microenvironment (19, 98). In this context, the PG domain may act as an intrinsic buffer due to the large number of COOH side groups in this region (19).

Since an acidic microenvironment is commonly observed in cancers, methods are needed to overcome low pHe drug resistance in order to improve therapeutic efficacy (99). Targeted treatment with proton pump inhibitors (PPIs) disrupt transmembrane pH gradients and can inhibit growth in human B cell leukemia (100). Also, inhibition of CAIX catalytic activity using monoclonal antibodies or specific small molecule inhibitors leads to intracellular acidification with the consequent impairment of the tumor growth and reduced invasive capacity of the tumors (101, 102).

Alternatively, acidosis can be inhibited directly with the ingestion of alkaline buffers, such as bicarbonate (103). Alkalization of tumors by administration of bicarbonate in drinking water effectively increased the extracellular tumor pH without affecting the pHi or systemic

pH and led to reduced metastases in human breast and prostate cancer mouse models and increased survival (104). Interestingly, immunohistochemical staining for CAIX in regions of ductal hyperplasia of the C57BL/6 TRAMP (transgenic adenocarcinoma mouse prostate) model showed increased expression in controls versus the group that had 200 mM sodium bicarbonate in their drinking water (105). However, these results do not generalize across all tumor cell lines, as fast-growing tumor cell lines, e.g. B-16 melanoma, showed resistance. A concern about buffer therapy is whether or not a balance can be reached between the acid load produced by the tumor and the quantity of bicarbonate that can be administrated. Thus the effectiveness of this therapy will be reduced in large tumors (106). However, the strong advantage of this strategy would be to restore a more neutral tumor pHe that should slow down the metastatic process and diminish drug resistance (77). Another approach that takes advantage of low tumor pHe involves the development by many groups of low pH activated micelle systems that penetrate into solid tumors and release their cytotoxic therapeutic payloads within the acidic microenvironment. However, additional *in vivo* studies are needed to determine their efficacy (107, 108).

5. CAIX expression in cancer

The use of CAIX as a prognostic indicator in oncology makes it a potentially important biomarker for the evaluation of tissues. CAIX is constitutively up-regulated in several cancer types such as the lung (109–111), colon (112, 113), colorectal cancer (114), gastric cancer (115), pancreatic cancer (116), breast (117–121), cervix (122–125), bladder (68, 126), ovaries (127), brain (128, 129), head and neck (130–132), astrocytomas (133) and oral cavity (134–136). The mechanism of upregulation has been determined in some cancers, e.g. clear cell carcinoma of the kidney, where the VHL gene is mutated leading to high levels of CAIX expression (up to 150-fold) as a consequence of constitutive HIF activation (37, 137–140).

High expression of CAIX has been shown to generally correlate with poor prognosis and is related to shorter disease-free and disease specific survival [reviewed (28)]. For example, CAIX is particularly highly expressed in the aggressive basal-like or triple negative breast cancers, a tumor subgroup with poor prognosis and known resistance to systemic therapy (119, 141), and high expression has been reported in patients with squamous cell carcinoma of the lung (111). However, there is still much to be uncovered regarding the prognostic indications of CAIX in various cancers. For example, it has been reported that CAIX over-expression, as detected by IHC staining, may be an indicator of a good prognosis in clear cell renal cell carcinoma (142). While, in other renal tumors such as chromophobe renal cell carcinoma and oncocytoma, CAIX is reported to be absent via IHC (143). In other cases, such as squamous cell carcinoma, IHC of CAIX does not appear to add prognostic or predictive value (144). Therefore, it is important to carefully characterize the prognostic value of CAIX in individual tumor types. Once established, the utility of CAIX expression in IHC has been shown to be a useful marker (145).

A correlation between CAIX expression and metastatic disease has been also observed. For example, a recent study that examined over 3600 human breast cancers reported that CAIX expression in distant metastases is an independent biomarker of poor prognosis (141). These

results were similar to other studies (122–124, 146, 147). A high level of CAIX expression in cervical cancer tumors is associated with the presence of lymph node metastases (124, 125). In addition, high stromal CAIX expression was associated with nodal metastasis and decreased survival in patients with surgically-treated oral cavity squamous cell carcinoma (148).

CAIX is also reported to be a useful IHC biomarker for the quantification of response to therapy. For example, CAIX has been used to predict renal cell carcinoma response to IL-2 therapy (149) and epirubicin/tamoxifen therapy in breast cancer (150).

Another membrane associated, CA, CAXII, is co-expressed with CAIX in several tumor tissues and highly expressed in a number of cancer types, including breast cancer (26, 151). Our group has recently reported co-expression of CAIX and CAXII in different types of breast cancer (152)(Figure 4). CAIX and CAXII staining was distributed in the cell membranes of tumor tissues. Interestingly, some samples were positive only for CAIX, some only for CAXII, and others had expression of both markers. This can be problematic when using agents that bind non-specifically to the CA active site if the goal is to target only CAIX (see discussions below). Lymph node metastases samples were positive for CAIX (71%) and CAXII (76%), both markers were expressed in 44% of the samples, and all of the positive lymph nodes were found to express either CAIX or CAXII, i.e., the combination of CAIX and CAXII covers 100% of patients with ALN metastases represented in our TMA. Heterogeneity of expression was found in 29% and 35% of the tumor samples for CAIX and CAXII, respectively, i.e. different areas of the same tumor on a histological section having different expression patterns for a given marker (152). We have planned future studies to determine if there is a correlation of CAIX expression with negative patient outcomes.

5.1 Detection of CAIX expression in tumors and metastases

The extracellular domain of CAIX, including PG and CA domains, can be released into the culture medium of CCRCC (clear cell renal cell carcinoma) cells or in the body fluids (blood, urine) of patients with CCRCC. This release is most likely a result of proteolytic cleavage. The soluble 50/54 kDa form is termed s-CAIX and it is cleared from the blood within a few days after nephrectomy (153). The concentration of s-CAIX is very low in healthy subjects (153, 154). Therefore, it can be assayed by ELISA in serum, plasma and tissue for clinical detection and prognostic evaluation of patients with different types of cancers. ELISA could be a potential quantitative method for non-invasive diagnosis of renal tumors. Two different groups have reported similar values for serum s-CAIX in CCRCC (114.0–126.1 pg/mL), higher than in other types of renal cancer and healthy samples (143, 154). Serum CAIX levels were significantly higher in CCRCC than in non-CCRCC and increased in conjunction with increased stage of tumor progression (154). Serum s-CAIX levels also correlated with the tumor size in CCRCC patients (143). In addition to serum s-CAIX, *CA9* mRNA may also be a promising molecular marker of CCRCC. For example, *CA9* mRNA was detected in 97% of CCRCCs and highly correlated with IHC staining of corresponding samples (155).

Similar studies have also been performed for other cancer types. For example, urine was tested from 23 patients with transitional cell carcinoma (TCC) or squamous cell carcinoma

(SCC) of the urinary bladder and renal pelvis, and the presence of urine s-CAIX correlated with tumor CAIX in most cases. Additionally, s-CAIX was detected in the urine of two patients with a suspected, but unconfirmed bladder tumor, who later developed tumors within six months of testing (156). However, this correlation was not observed in TCC patients. Preoperative serum concentrations of CAIX were reported to be significantly higher in patients with high intratumoral expression of CAIX in vulvar cancer (157). Serum levels of CAIX in metastatic breast cancer and vulvar cancer were correlated with poor prognosis (157, 158). In non-small cell lung cancer, high plasma levels of s-CAIX were determined to be an independent prognostic biomarker, particularly for early-stage I or II carcinomas and were associated with significantly worse survival (111). Also, detection of s-CAIX protein by ELISA and immunocytochemistry have been used to detect malignant pleural effusions (145). The potential use of serum s-CAIX as a biomarker for therapy response was tested in patients with primary epithelial ovarian cancer, yet were not significantly altered during first-line therapy (159).

As mentioned above for CCRCC, immunohistochemical (IHC) staining may be also used to detect CAIX in tumor samples (145). IHC of CAIX is an emerging field of interest and holds a great deal of promise for quantification of disease progression, use as a predictive or prognostic indicator, and as an indicator of therapy response. Like other IHC markers, it can be used to evaluate the expression or localization of CAIX in tissue. Using standard color producing enzymes (i.e. peroxidase coupled with DAB) and amplification techniques common to the practice of IHC staining, CAIX can be visualized in a range from negative to strong positive staining intensity. CAIX is generally regarded as a specific IHC biomarker in a myriad of tissue types (117). As expected, CAIX is generally localized on the cell membrane but may also be found to a lesser degree in the cytoplasm.

6. Targeting of CAIX (imaging and therapy)

6.1 CAIX imaging

The characterization and detection of hypoxic regions within solid tumor masses is an important issue in cancer care because hypoxia is an independent predictor of poor survival in several cancer types and hypoxic cancer cells are less sensitive to chemo- and radiation-therapies (160). Therefore, a molecular imaging approach based on selective ligands to accessible proteins overexpressed at sites of hypoxia is desired. Such an agent could help physicians to decide which patients will benefit from adjunctive anti-hypoxia therapy (61, 161). Also as discussed above, since CAIX has high and broad expression among a number of aggressive and late stage types of tumors and metastases, the non-invasive imaging of CAIX expression has potential for use in cancer diagnosis and staging, and to detect and follow the response of disseminated metastatic disease to systemic and targeted therapies. Both antibodies and small MW compounds have been used for targeted imaging of CAIX expression (28).

6.1.1 CAIX specific monoclonal antibodies for imaging—G250 was characterized as a monoclonal antibody in the 1980s by Oosterwijk et al. (14), and was shown to be a very strong biomarker for CCRCC due to its absence in normal kidney tissue. The specific epitope for the G250 antibody is unknown, but it has excellent specificity for CAIX in

immunohistochemical analyses. To reduce human anti-mouse antibody responses (HAMA), a chimeric version of G250 (cG250) was developed in 1997, and was recently radiolabeled with iodine-124 or zirconium-89 for diagnosis, and with iodine-131 or lutetium-177 for therapy (162, 163). Phase I of human clinical trials of positron emission tomography/computed tomography (PET/CT) imaging of CCRCC with Iodine-124 labeled cG250 have shown excellent sensitivity, specificity, positive predictive values, and negative predictive values (94,100, 100, and 90%, respectively) (164). Hence, CAIX targeted PET/CT-based molecular imaging has potential to alter the care and welfare of patients suspected of having CCRCC and could be used to monitor patients with a history of CCRCC. In addition, this approach allows for the non-invasive and non-surgical differentiation of clear cell and non-clear cell RCC. Currently, Wilex AG (Munich, Germany) is conducting phase III clinical trials using this PET imaging agent, under the name of Redectane®. Wilex is also developing a therapy application of G250 under the product name Rencarex® (165).

The M75 antibody is highly specific for CAIX (166). It recognizes the extracellular proteoglycan-like domain and is useful for western blotting, immunoprecipitation, and immunohistochemistry. A radiolabeled derivative has been developed for pre-clinical imaging of CAIX in hypoxic tumors in mouse xenograft models (167, 168) and CAIX selective accumulation of the M75 antibody labeled with iodine-125 was demonstrated in HT-29 xenografts in nude mice (167).

Fully human CAIX single-chain variable fragment (scFv) mini-antibodies, or minibodies, named A3 and CC7 were generated using phage-display technology by Ahlskog et al. (169). These minibodies recognize the extracellular carbonic anhydrase (CA) domain of human CAIX and have low nanomolar affinity to CAIX. Because they are fully human, A3 and CC7 are expected to be non-immunogenic in patients with cancer. Also, because of their smaller size, approximately a factor of 10 smaller compared to full-length antibodies, these minibodies are expected to have faster tissue distribution and clearance relative to full-sized antibodies. The A3 and CC7 minibodies do not inhibit CAIX activity and do not bind to CAXII, which shares 39% sequence identity with CAIX. We have reported that at least one of the two cell-surface markers, either CAIX or CAXII, is expressed in 100% of breast cancer axillary lymph node (ALN) metastases on our TMA.

We have also developed CAIX and CAXII specific monoclonal antibody-based near-infrared fluorescent (NIRF) molecular imaging probes (CAIXAb-680 and CAXIIAb-68) using antibodies from R&D Systems for the non-invasive detection of breast cancer ALN metastases and we have confirmed the high selectivity of the probes *in vitro* and *in vivo* using preclinical breast cancer metastasis models (152) (Figure 5).

6.1.2 Imaging CAIX expression using small molecule-based probes—Carbonic anhydrase inhibitors are divided to four groups: inorganic anions, sulfonamide based compounds (sulphonamides, sulphanilamides, sulphamates and their derivatives), phenols and coumarins (1, 170–172). Inorganic anions and sulfonamides inhibit CAIX by coordinating to the zinc ion within the active site, with mM to μM Ki inhibition (the anions) or μM to nM Ki inhibition (sulfonamides and their congeners) (1, 170, 173–175). Phenols are generally low μM Ki CA inhibitors, and act by anchoring to the zinc-bound water

molecule/hydroxide ion through a network of hydrogen bonds which stabilize the enzyme-inhibitor adduct (171). Coumarins are a novel class of CA inhibitors and act differently compared to the other inhibitors, i.e. through a non-zinc mediated mechanism. Coumarins function as suicide inhibitors, undergoing hydrolysis to 2-hydroxycinnamic acids and anchoring irreversibly at the entrance to the active site cavity (172). Although several low nanomolar and isoform-selective CAIX inhibitors based on the coumarin/thiocoumarin ring were recently reported (172), the best investigated and most robust class of inhibitors are the sulfonamides due to their high affinity, availability, and ease of chemical manipulation (1, 176). Supuran's group has pioneered the development of specific CAIX inhibitors to target tumor growth and many sulfonamide CA inhibitors with low nM Ki inhibition have been identified in the last several years, demonstrating particular promise as potential anti-cancer agents (1, 94, 177, 178).

Interestingly, it has been demonstrated by testing several cell lines in hypoxic conditions, that sulfonamides bind to CAIX only when the enzyme is active, indicating that the active site of CAIX is only available for sulfonamide binding during hypoxia (94, 179). Hence, CAIX sulfonamides can distinguish cells that are currently in hypoxic conditions from those that were previously hypoxic, while CAIX specific antibodies do not, which is a big advantage since CAIX expression is very stable (with a half-life of ~40 h) with CAIX remaining on the cell surface for a relatively long time after reoxygenation (76, 94). Therefore, imaging with CAIX inhibitors might provide different imaging and prognostic information compared to monoclonal antibodies (98).

However, due to a high degree of homology among CA isotypes, sulfonamide-based inhibitors are generally not specific to the CAIX isoform, i.e. sulfonamides specifically bind to the catalytic sites of other CAs (1, 180). This is a problem, because unmodified sulfonamides are able to pass through the plasma membrane and can thus interact with the physiologically dominant CAI and CAII isoforms which are soluble proteins located in the cytosol or mitochondria. Several strategies are being employed to develop selective CA-isozyme inhibitors without "off-target" inhibition of intracellular CAs. These strategies include the addition of charged species or bulky entities such as FITC, albumin or hydrophilic sugar moieties, all of which prevent transportation across the plasma membrane (166). However, these strategies do not solve the problem of extracellular isoforms other than CAIX that are known to be overexpressed in cancers, e.g. CAXII. In fact, both CAIX and CAXII are transmembrane proteins with catalytic domains oriented extracellularly. Hence, the development of CAIX specific inhibitors is an area of significant interest and the design and synthesis of CAIX selective inhibitors has been recently reviewed (180, 181). For example, sulfonamides conjugated to fluorescent dye (FITC) have been developed and are shown to have membrane-impermeant properties and high affinities for CAIX. These agents have been shown to bind to cells that express CAIX in hypoxic conditions, but not in normoxic conditions or to control cells that lack CAIX expression (94). This finding suggests that hypoxia may alter the conformation of the CAIX active site, allowing access to the inhibitor. It has been proposed that interaction of the highly acidic PG domain with highly basic residues around the conserved zinc-binding histidines form a cover at the entrance to the catalytic site that open in hypoxic conditions. In support of this hypothesis, it

was observed that a CAIX variant that lacks the PG domain binds to inhibitor in normoxic conditions at high levels that are comparable to binding in hypoxia (178).

In vivo studies with fluorescent sulfonamides have also shown promise for detecting CAIX expression in HT-29 and SK-RC-52 tumor xenografts (178, 182). An inhibitor-fluorescent dye conjugate was also shown to inhibit the CAIX-mediated acidification of the tumor extracellular environment (141, 179, 183). In addition, Dubois et al. reported significant accumulation of the inhibitor in the HT-29 colorectal tumors when animals were placed in hypoxic breathing conditions (7% oxygen). Furthermore, the bound CA inhibitor fraction rapidly and significantly decreased upon return to normoxic conditions and tumor reoxygenation (177).

Also very recently, a series of acetazolamide derivatives conjugated to various near-infrared fluorescent (NIRF) dyes were synthesized with the aim of imaging hypoxia-induced hCAIX expression in tumor cells *in vitro*, *ex vivo* and *in vivo*. The compounds demonstrated up to 50-fold selectivity for hCAIX compared to the cytosolic hCAII isoform. Some of the compounds also showed inhibition selectivity for other transmembrane isoforms as well, e.g. hCAXII and hCAXIV. A pilot *in vivo* study in HT-29 tumor bearing mice quantified significant accumulation (10% i.d.) in the tumor with little accumulation in other organs (except the kidneys), demonstrating the potential use of these new compounds for quantitative imaging of CAIX via fluorescence molecular tomography (FMT) (184). These results illustrate the potential of NIRF CAIX inhibitors and FMT imaging for pre-clinical studies to non-invasively quantify CAIX expression as an endogenous marker of tumor hypoxia. This capability is crucial to the study of the underlying biology of tumor hypoxia and the development and monitoring of novel anti-cancer therapies (184).

A 99mTc-labeled sulfonamide derivative has been synthesized and evaluated for visualization of CAIX expression by single photon emission computed tomography (SPECT) imaging. However despite the favorable Ki values of the tracer, a maximum tumor tracer uptake of only 0.1% ID/g was observed at 30 min post injection, indicating that the tracer is not very promising for imaging of CAIX (185). Although tracer uptake in tumors was minimal, tumor-to-blood ratios increased as a function of time and a different radionuclide with a slower decay rate may produce greater contrast at later time-points.

6.2 CAIX targeted therapy

CAIX is highly expressed in different tumor types and has relatively low expression in normal tissues; has an important role in tumor progression, acidification and metastasis; and is located on the extracellular surface of cell membranes, allowing for efficient targeting by antibodies or small molecule inhibitors. Therefore, CAIX constitutes an attractive and promising candidate marker for systemic anticancer therapy that targets disseminated metastatic disease. Two major therapeutic tools are being studied for targeting of CAIX: monoclonal antibodies and small molecule inhibitors [reviewed by (28)].

6.2.1 CAIX specific monoclonal antibodies for therapy—CAIX-specific monoclonal antibodies can be useful for anti-tumor therapy either by (a) antibody-dependent cell cytotoxicity (ADCC); (b) targeting the CAIX active site; or (c) receptor-mediated

internalization for targeted delivery of various therapeutic agents, including cytotoxins and radionuclides (28).

a. The first category of antibodies acts through ADCC. For example cG250 which targets the PG-like domain of CAIX and is marketed by WILEX AG under the trade name RENCAREX® has been extensively characterized as an anticancer immunotherapy [90]. This antibody acts via ADCC (186), and phase I and II trials have demonstrated safety and high efficacy of the antibody in either monotherapy or in combination with interferon (IFN)-α for treatment of renal cell carcinoma (RCC) (187, 188). Phase III trials have just begun using cG250 as an adjuvant therapy for reducing recurrence in surgically-treated renal cell carcinoma (RCC) patients who have a high risk of relapse and in combination with interleukin 2 (IL-2) or IFN-α for metastatic RCC (28, 187).

b. While cG250 is directed at the PG-like domain, recent studies have focused on developing antibodies that target the catalytic domain of CAIX, thus targeting its tumorigenic functions, including pH regulation (28). For example a mAb directed against the CAIX catalytic domain has been generated using hybridoma technology and this antibody demonstrated efficient binding, internalization and persistence in cultured cells. *In vivo* evaluation of the antibody demonstrated an anti-cancer effect in a mouse xenograft model of colorectal carcinoma by limiting tumor growth. However, the effect was not observed when tumors were allowed to grow and become established prior to treatment (189). Though this might be the expected result, since buffer therapy has no effect on the growth of established primary cancers, while exerting a significant inhibition on the development of spontaneous metastases (104).

Antigen-binding antibody fragments (Fab) against human CAIX have been generated by phage-display and inhibition of CAIX demonstrated in spheroid cell cultures, suggesting that these Fabs could be used to inhibit CAIX therapeutically (190). Two mAbs with high affinity to CAIX generated by phage display have also been described (169). However more studies are needed for preclinical evaluation of these antibodies.

c. The third category of CAIX antibodies are those that are internalized and can thus be used for delivery of therapeutic payloads, such as radionuclides and cytotoxic agents. For example, stability, biodistribution and therapeutic efficacy of various radio-immunoconjugates of cG250 have been tested in nude mice with subcutaneous renal cell cancer (RCC) tumors and high therapeutic efficacy has been demonstrated by attachment of (177)Lu, (90)Y or (186)Re compared to (131)I-cG250 (191). A phase I/II trial in metastatic RCC patients is currently ongoing for (177) Lu-cG250 (192). Also in another study a CAIX mAb which was identified by phage display was conjugated to monomethyl auristatin E (a synthetic antineoplastic agent) and high efficacy as a potent anti-tumor drug confirmed in several preclinical human xenograft tumor models (193).

Alternative combination therapies are being designed that include targeting of CAIX. An example is the tyrosine kinase inhibitor sunitinib (Sutent®). This approved first line therapy

for metastatic renal cell carcinoma (mRCC) has had limited clinical success. Oosterwijk et al., are developing an agent that combines Sutent® with targets of CAIX for enhancement of therapeutic effect. A second example involves treatment of the same disease (mRCC) with autologous T-cells genetically retargeted against CAIX (194).

6.2.2 CAIX targeted small molecule inhibitors for therapy—Sulfonamides are commonly used as diuretics, anti-glaucoma, anti-obesity, and anti-infective treatments. Additionally, they have potential for use as antitumor drugs and this is an active area of clinical research. In fact, with the help of X-ray crystallography, much recent progress has been made for the designing of selective CAIX inhibitors. For example, Indisulam, a sulfonamide which was recently investigated in phase II clinical trials, is considered one of the most potent anticancer sulfonamides yet and has demonstrated high anti-tumor activity in various preclinical cancer models (195). The combination of CAIX inhibitors with conventional chemotherapy may yield even better efficacy (98). Also, one of several potent bis-sulfonamide CAIX inhibitors identified by screening 1 million compounds in a DNA-encoded chemical library has demonstrated high and specific accumulation in tumor models (196). In addition, due to high-expression in hypoxia, CAIX is an attractive target for the design of hypoxia-activatable prodrugs. This may lead to a completely new approach for developing less toxic anticancer agents [see Ref (181) for review]. Recently, carbonic anhydrase inhibitors coated with gold nanoparticles, which are membrane impermeant and therefore more effective in targeting the extracellular active site of CAIX, have been developed (197). These nanoparticles exhibited more potent inhibition of CAIX compared to acetazolamide, and may be a candidate for RCC treatment or imaging and treatment of hypoxic tumors. Targeting of CAIX by small molecules in preclinical models of human cancer has been reviewed extensively in ref (7, 28).

7. CAIX and CAXII selectivity issues

CAXII is a transmembrane isoform with an extracellular active site that was originally identified because of its overexpression in renal cancer cells (43, 198). CAIX and CAXII proteins share an overall amino acid sequence identity of 35% and there is considerable homology at the catalytic site with the three key zinc-binding histidine residues conserved in both sequences (43). As mentioned above, CAIX contains an additional proteoglycan-like domain that has been implicated in cell adhesion, which is not present on CAXII (17, 20, 22). The lack of PG domains suggests that CAXII has a more neutral pH optimum for activity.

Similar to CAIX, CAXII is highly overexpressed in breast cancer (26) and other tumor types [for review see (77)], but it is also expressed in some normal tissues such as pancreas, kidney, gut and the gastrointestinal tract (199–202). Several studies have shown that while CAIX expression is associated with a negative prognosis, including resistance to chemo- and radiation-therapy in breast cancer (117, 203, 204), CAXII has been associated with a favorable prognosis in invasive breast cancer (205).

Although expression of both CAIX and CAXII can be induced by hypoxia (29), CAXII is not as robustly regulated by hypoxia as CAIX and, if present, its HIF response element

(HRE) has not been identified (26, 29, 54). Thus, the mechanism for CAXII induction by hypoxia is still in question. Our group has studied CAIX and CAXII expression in breast cancer tumor xenografts using multiple tumor lines. CAIX was invariably induced in regions adjacent to necrosis, while CAXII induction was generally lower compared to CAIX and that, although cell line dependent, both markers could have significant constitutive expression distal to the necrotic edge (Figure 6).

Thus, imaging probes that specifically target CAIX may provide superior results compared to probes that non-specifically target the CA active site and can cross-react with expression of other cell-surface CA isoforms, e.g. CAXII. However in terms of therapeutic agents, the use of membrane impermeable sulfonamides, which display inhibitory constants in the low nanomolar range both towards CAIX and CAXII may be useful; because of high expression of both CAIX and CAXII is observed in several cancers (55, 152). Simultaneous silencing of both CAIX and CAXII in transfected LS174T colorectal cancer xenograft models led to substantial growth retardation that was greater compared to HIF-1α silencing alone, and greater than knockouts of CAIX or CAXII individually, which yielded substantially lower tumor growth retardation (54). Therefore drugs that can target both CAIX and CAXII may act as novel and potentially efficient drug approaches (55). However, CAXII expression in normal tissues involved in agent clearance (e.g. kidney) and toxicity (e.g. pancreas and gut) is of some concern in the context of therapy.

Specific peptides could be a promising candidate for imaging of hypoxia. Peptides present an attractive alternative to antibodies due to their small size and therefore optimal pharmacokinetic properties and high effectiveness of tumor penetration. In addition, peptides do not possess the immunogenic potential of antibodies and are easier and less expensive to produce (206). Recently, a new peptide (CaIX-P1) with specificity for the extracellular domain of CAIX was identified by phage display technology using a commercially available Ph.D.12 library and the CAIX binding affinity and biodistribution studied (207). Although the stability and affinity of CaIX-P1 was recently optimized, organ distribution studies revealed low tumor-to-blood ratios and high background distribution, which is not favorable for imaging applications (208). Therefore, further research is required for generation of peptide-based ligands with high target specificity for human carbonic anhydrase IX.

8. Conclusion

In summary, CAIX is considered to be a promising marker for targeted diagnostic imaging and systemic anticancer therapy due to its overexpression in many cancers and metastases with a limited presence in their normal tissue counterparts. CAIX expression is generally associated with a poor prognosis and is associated with tumor progression to metastasis due to its role in acidification of the extratumoral medium, which leads to both the acquisition of the metastasic phenotype and chemoresistance to weakly basic anticancer drugs.

Inhibition of CAIX activity by either specific antibodies or small molecules has been studied in the preclinical setting and more recently in clinical trials as a mode of therapy. Many classes of CAIX targeted therapeutic agents have been developed and pharmacological

evaluation has demonstrated promising results. Some of these agents are now in phase III clinical trials. Concerns remain about off-target toxicities of agents that are not excluded to the extracellular environment due to interactions with intracellular CAs, and due to lack of selectivity among other extracellular CAs, e.g. CAXII which is expressed in normal tissues of concern.

There is significant interest in the development of diagnostic imaging tools for the non-invasive detection of tumor hypoxia via CAIX induced expression, for detection of CAIX expression in lymph node metastases for staging, for the diagnosis of disseminated disease and to follow response to systemic therapy during treatment of distal metastasis. In conjunction with the development of targeted therapies, there is now significant interest in the pharmaceutical industry in the development of companion targeted imaging agents for use during clinical trials. In terms of the use of CAIX as a marker for tumor hypoxia, it has been shown that CAIX is generally up-regulated in regions of hypoxia. However expression of CAIX in non-hypoxic regions of tumors has also been observed due to HIF-1 stabilization in cancer. Also, high constitutive CAXII expression in tumors and metastases may be a confounding factor for imaging agents that are not CAIX specific. However, for detection of elevated CA expression in disseminated disease, this requirement for specificity is not as important. Although, since CAXII expression is also observed in normal tissues, this could cause increased non-specific background signal in agents that are not CAIX specific.

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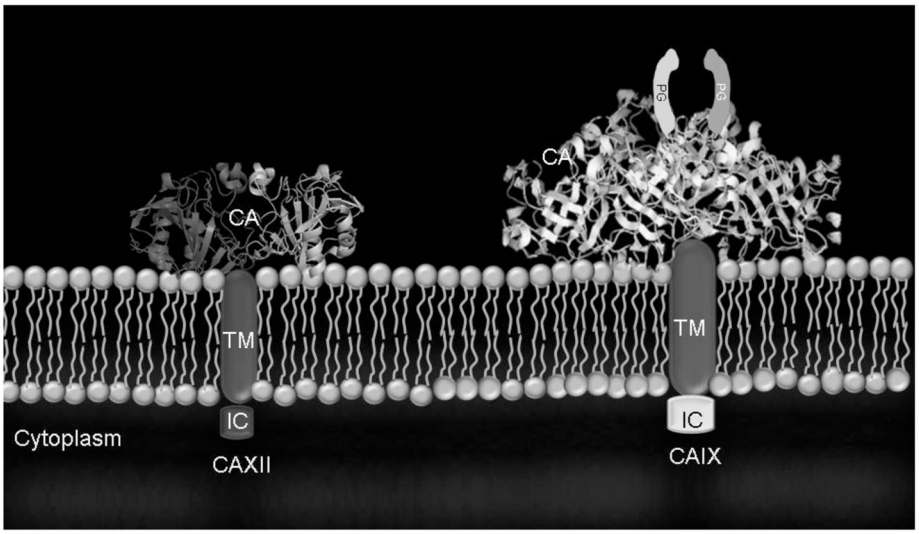

Figure 1.

Proposed model showing the structural arrangement of the CAXII and CAIX dimer on the cellular membrane (23, 24). PG= proteoglycan-like domain, CA= catalytic domain, TM= transmembrane segment, IC= intracellular, cytosolic tail.

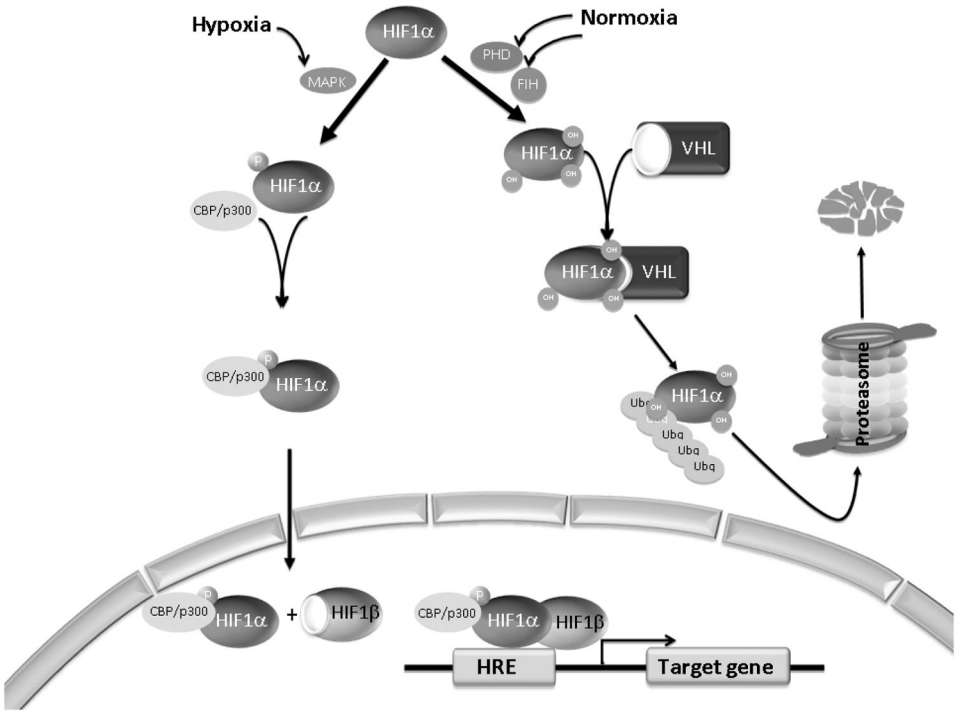

Figure 2.

Hypoxia-induced gene expression mediated by the HIF1 transcription factor heterodimer. HIF-1α is degraded in normoxia by the ubiquitin-proteasomal pathway. In hypoxia, it is phosphorylated by MAPK and bound by CBP/p300, inducing heterodimirezation with HIF-1β and transcription of variety of genes.

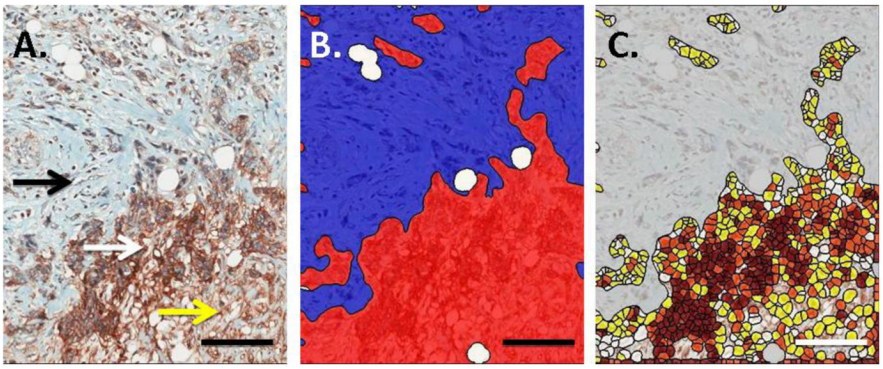

Figure 3.

Immunohistochemical staining of CAIX is an important tool to investigate 2-D localization of expression. A) Dark brown CAIX overexpression at the edge of the tumor (white arrow) contrasts not only with the adjacent microenvironment in blue (black arrow), but also with the decreased expression of the tumor >200microns from the edge (yellow arrow). B) A mask depicts regions selected by computer learning which segment and classify the tumor (red) and non-tumor (blue) regions. C) Single cell segmentation and classification of the tumor cells demonstrates the location of strong (red), moderate (orange), weak (yellow) and negative (white) expressing cells. These classifications allow investigators to quantify metrics of the IHC stain localization. Scale = 150 microns.

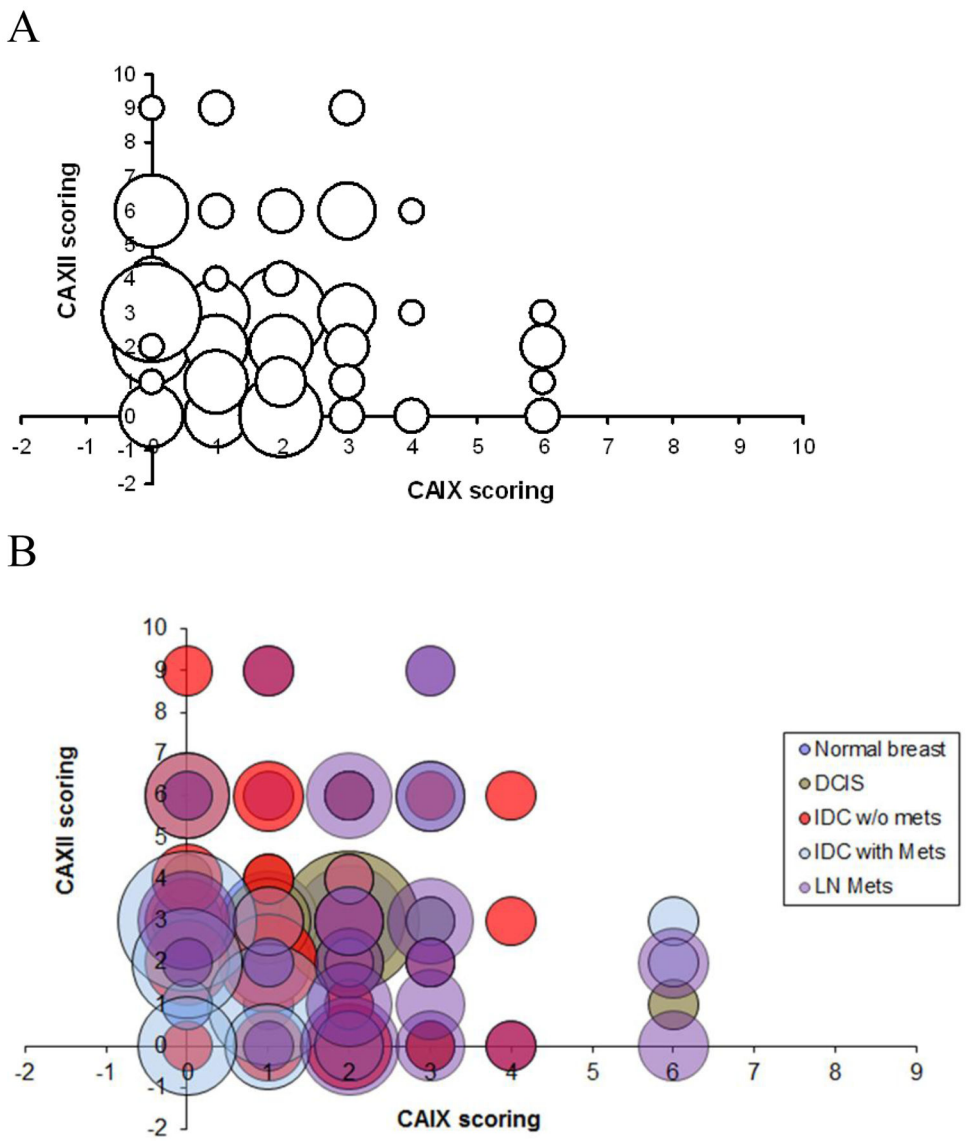

Figure 4.

Bubble plot of pathologist scores for CAIX and CAXII IHC staining A) for samples from all breast cancer patients on the tissue microarray (TMA), n = 180; or B) for 47 normal breast tissues, 42 ductal carcinoma in situ, 43 invasive ductal carcinomas without metastasis, 46 invasive ductal carcinomas with metastasis and 49 lymph node macrometastases of breast cancer on the TMA.

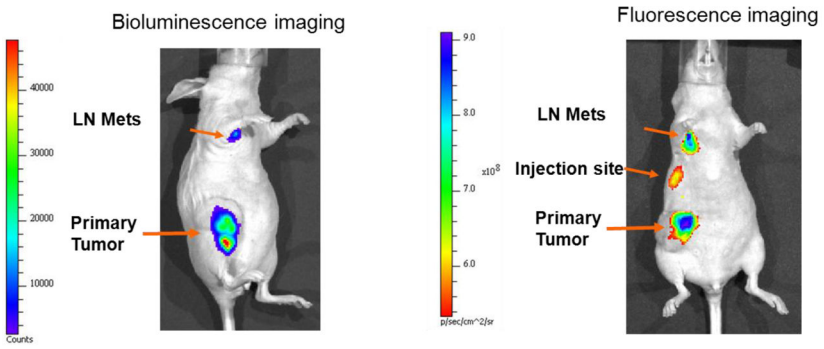

Figure 5.

In vivo bioluminescence and fluorescence images of a mouse following spontaneous metastasis to the axillary lymph node from a primary mammary fat pad (MFP) xenograft tumor. MDA-mb-231 cells were used that were engineered to overexpress luciferase and constitutively express CAIX. The fluorescence image was acquired 24 h after MFP injection of CA9Ab-680 probe.

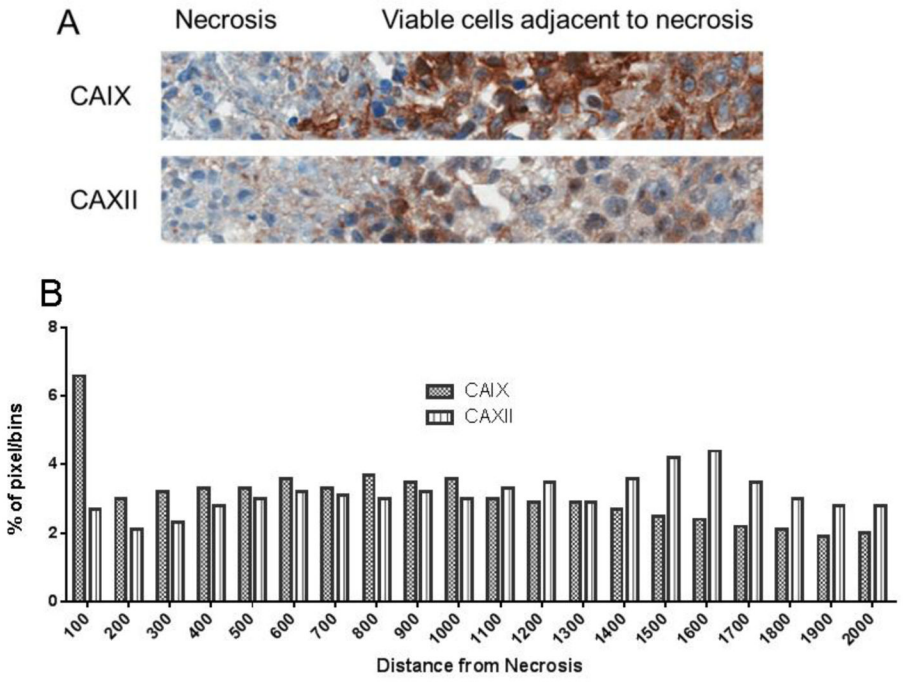

Figure 6.

A) CAIX and CAXII IHC staining in a region of a representative MCF-7 tumor xenograft adjacent to necrosis. B) Quantified staining intensity by distance from necrosis with pixels 0–100 being adjacent to a necrotic region.