# REVIEW

# Is carbonic anhydrase IX a validated target for molecular imaging of cancer and hypoxia?

Jianbo Li[‡,1,2], Guojian Zhang[‡,1,2], Xuemei Wang[*,1] & Xiao-Feng Li[*,2]

**ABSTRACT**   The presence of hypoxia is a general feature of most solid malignancies, and hypoxia is considered as one of major factors for anticancer therapy failure. Carbonic anhydrase IX (CAIX) has been reported to be an endogenous hypoxia marker, CAIX monoclonal antibodies, their segments and inhibitors are developed for CAIX imaging. However, growing evidence indicates that CAIX expression under hypoxia condition may be cancer cell lines or cancer-type dependent. Here we review the current literature on CAIX and discuss the advantage and limitation of CAIX as a target for tumor hypoxia imaging. Accordingly, CAIX would be unreliable as a universal target for cancer and tumor hypoxia visualization.

In 2013, approximately 1,660,290 new cancer cases are diagnosed and 580,350 patients are died of cancer in the USA [1]. Hypoxia is a common feature of large primary solid malignancies and is recognized as a negative determinant of clinical outcome [2–9]. It is now established that tumor hypoxia is an important determinant of relapse-free survival and overall clinical outcome [4,10–11]. This appears to be largely independent of the treatment modality used and implies that hypoxia is associated with a more aggressive tumor phenotype. There seems to be at least three distinct manifestations of the hypoxic phenotype: Hypoxic cells are more resistant to ionizing radiation and chemotherapy than aerobic cells; The hypoxic environment is selective for genetically unstable tumor cells, and hypoxia is associated with tumors that are more likely to metastasize [10,12–13]; and hypoxia regulates the level of HIF-1$\alpha$, which controls the expression of genes that are involved in oxygen and glucose supply and utilization [14]. Therefore, it is important to detect the hypoxic status for solid cancers.

Recently multiple biomarkers related to tumor hypoxic microenvironment have been discovered and offered as targets for cancer detection, treatment and monitoring, such as HIF-1 [15–17], VEGF and VEGF receptors [18–20], glucose transporter 1 [21–23] and carbonic anhydrase IX (CAIX) [24–29]. CAIX is strongly induced by hypoxia through the HIF-1 transcription factor, and has reported to overexpress in some types of cancer [30,31]. Promising results for using anti-CAIX antibodies and CAIX inhibitors for tumor hypoxia imaging have been reported [32–34].

However, growing evidence indicates that the CAIX expression under hypoxia conditions in cancer cell lines is cancer cell lines or cancer types dependent [30], and some cancer cell lines have undetectable CAIX expression under hypoxia condition [35,36]. In addition, re-oxygenated (previously hypoxic) cancer cells may still show high level of CAIX expression [36]; CAIX has a half-life up to 2–3 days under normoxic condition. In contrast, acute hypoxic cancer cells may not express detectable CAIX.

[1]Department of Nuclear Medicine, Inner Mongolia Medical University Affiliated Hospital, Hohhot, Inner Mongolia, China
[2]Department of Diagnostic Radiology, University of Louisville, 530 S Jackson Street, CCB-C07, Louisville, KY 40202, USA
*Authors for correspondence: Tel.: +1 502 217 8285; Fax: +1 502 852 1754; linucmed@gmail.com; wangxuemei201010@163.com
‡Authors contributed equally

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The open question is whether CAIX is a reliable and universal target for cancer treatment and especially tumor hypoxia visualization. In this review, we focus on the topic of whether CAIX is a validated target for molecular imaging of cancer and hypoxia.

## Carbonic anhydrases

Carbonic anhydrases (CAs) are a large family of zinc metalloenzymes that catalyze the reversible interconversion of carbon dioxide and water to bicarbonate and a proton. There are 16 different alpha-CA isoforms currently known, differing in tissue distribution, subcellular localization and molecular biophysical properties. They are involved in diverse physiological processes, including acid-base regulation, respiration, ion transport and bone resorption [37].

CAIX, a CAs family member, is a transmembrane protein and was first detected in human cervix carcinoma HeLa cells and initially named the MN protein in 1992 by Pastoreková et al. [38]. In 1994, Pastorek et al. identified CAIX by sequence analysis of its cDNA isolated using the monoclonal antibody M75 specific for a plasma membrane antigen in HeLa cells [39].

The overexpression of CAIX in normal human tissues is rare [40]: it has limited expression and distribution in a few normal tissues (mainly the GI tract), and this expression is either decreased or lost during carcinogenesis [41]; however, it is ectopically expressed in numerous tumors which are mostly derived from tissues that do not normally express CAIX [25,27–29]. For example, CAIX is overexpressed in clear-cell renal cell carcinoma because of a mutation in Von Hippel–Lindau tumor suppressor, but is expressed at low levels in normal kidney tissue. [42]. This atypical differential expression pattern enables researchers to designate CAIX as a tumor-associated protein and to use it as a biomarker of tumor.

CAIX is a membrane-associated glycoprotein [43], consisting of an extracellular catalytic domain, a proteoglycan-like region (PG domain), a transmembrane anchor and a short C-terminal cytoplasmic tail **(Figure 1)**. The PG domain, which is adjacent to catalytic domain and involves in $CO_2$ hydration at more acidic pH values, is the distinctive feature of CAIX differing from the other known CAs. Cancer cells maintain a near neutral intracellular pH by CAIX, thereby favoring cell-survival. The acidic extracellular environment activates metalloproteinases and release of growth factors, thus facilitating tumor invasion and metastases [44]. Pastoreková et al. [45] found CAIX also has a potential to decrease cell–cell adhesion by destabilization of E-cadherin links to cytoskeleton. In hypoxic circumstances, de-adhesion capacity and interaction with β-catenin may represent one important aspect of CAIX functionality, by which it may contribute to the acquisition of increased tumor aggressiveness.

## CAIX & hypoxia

Hypoxia is a characteristic feature of many solid tumors [46] and has been described in a variety of human malignancies, including prostate cancer [47], head and neck tumors [48], non-small-cell lung cancer [49], brain cancer [50] and breast cancer [51]. The hypoxic microenvironment is mainly due to an imbalance between oxygen supply and consumption, which is caused by the rapid proliferation of tumor cells resulting in increased diffusion distances between blood microvasculature and the tumor cells [31,52]. The impact of the hypoxic tumor microenvironment is multifaceted [46,53–54], including genetic instability, abnormal angiogenesis, limitless proliferative potential, evasion of apoptosis, invasiveness, survival and metabolism. Hypoxia-inducible factor HIF-1 has been verified as the main transcription factor that regulates the tumor cellular adaptation to hypoxia [55,56].

HIF-1 regulates the expression of many genes under hypoxic conditions through HIF-1 pathway [55–58]. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunit. HIF-1β is constitutively expressed, insensitive to changes in oxygen concentration, while HIF-1α is very sensitive to oxygen concentration. Under normoxic condition, HIF-1α undergoes proline hydroxylation and binds to Von Hippel–Lindau protein and is ultimately broken down after ubiquitination. Under hypoxic condition, HIF-1α is stabilized and translocated to the nucleus and heterodimerizes with constitutively expressed HIF-1β. The heterodimer then binds to the hypoxia-response elements sites of target genes, including CAIX, crucial to adapt to the hypoxic environment for the tumor cells. CAIX has thus been proposed as a reliable endogenous hypoxic tumor marker that may be helpful in clinical practice [27].

## CAIX-targeted preclinical imaging

The rare distribution of CAIX in normal tissues makes radionuclide labeled monoclonal
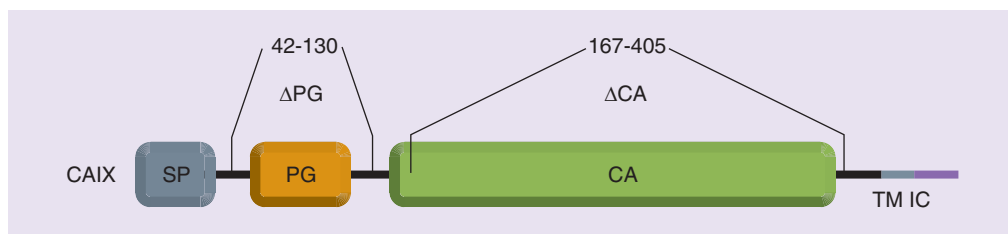
**Figure 1. Domain organization of the carbonic anhydrase IX protein.**
CA: Catalytic domain; IC: Intracellular cytosolic tail; PG: Proteoglycan-like domain; SP: Signal peptide;
TM: Transmembrane segment.
Reproduced with permission from: original published work by De Simone G, Supuran CT [43] © by
Elsevier BV (2010).

antibodies against CAIX or CAIX-specific small molecule inhibitors excellent biomarkers of hypoxia in many solid tumors in preclinical studies [25,59].

Zhang et al. [24] found that $^{125}$I-labeled monoclonal antibody against CAIX ($^{125}$I-MAb) could be used to visualize tumor hypoxia. The biodistribution at 24 h postinjection (p.i.) of $^{125}$I-MAb and planar imaging at 48 h p.i. showed specific tumor uptake in the mouse bearing xenografts of human colorectal cancer HT29 cells. Immunohistochemical staining confirmed prenecrotic overexpression of CAIX within the tumors. Carlin et al. [33] investigated the efficiency of intact and IgG fragments of cG250 against CAIX in mice with HT29 tumor and demonstrated that $^{111}$In-DO3A-F(ab')$_2$-cG250, a lower molecular weight antibody fragment, had a faster uptake into areas of high CAIX expression, but had a much lower absolute tumor uptake and reduced tumor to muscle ratios at 24 h p.i. $^{111}$In-DO3A-Fab-cG250, another fragment, had a similar nature with F(ab')$_2$ fragment in vivo. However, highest HT29 tumor uptake of intact antibody $^{111}$In-DO3A-cG250 happened 7 days p.i. Accordingly, radionuclide labeled anti-CAIX antibodies or IgG fragment could significantly concentrate in the high CAIX expressed HT29 tumors and maybe useful for cancer and hypoxia imaging in this specific cancer line. Of note, hypoxic cancer cells, including HT29 cells, had be observed to have a short lifespan of approximately 2 days in vivo [60] though in vitro cell culture studies demonstrated that hypoxic tumor cells can survive up to approximately 10 days at hypoxic/anoxic atmosphere [61–64]. Therefore, imaging conducted 2–7 days after inoculation of radiolabeled CAIX antibodies or IgG fragments unlikely reflect real time tumor hypoxia.

CAIX inhibitor imaging has been well documented in literature. Akurathi et al. [65] synthesized [$^{99m}$Tc(CO)$_3$ (L)](L=N-(pyridin-2-yl-methyl)-N[2-(4-sulfamoylphenyl)-ethyl]aminoethyl acetate) complex ($^{99m}$Tc-complex) and the corresponding rhenium congener (Re-complex). In vitro affinity studies revealed that Re-complex had a dissociation constant (Ki) of 58 nM for CAIX. Re-complex efficiently reduced CAIX-mediated acidification of extracellular medium in vitro efficacy. Though tumor to blood activity ratio increased from 0.65 (at 1 h p.i.) to 1.14 (at 2 h p.i.), biodistribution results showed that $^{99m}$Tc-radiolabeled complex had a low uptake in tumor tissue (only 0.13, 0.09, 0.08 and 0.05%ID/g at 0.5, 1, 2 and 4 h p.i.). Rami et al. [66] designed and synthesized several CAIX inhibitors, sulfonamide/sulfamide/sulfamate derivatives containing 2- or 5-nitroimidazoles moieties, which target the active site of CAIX. The 5-nitro-imidazole series at a concentration of 1 mM could significantly reduce extracellular tumor acidification in HT29 and HeLa cells, which overexpressed CAIX with treatment of hypoxia. Results from in vivo tests showed the tumors of mice bearing HT29 tumor needed longer average time (25 days) to reach 4× starting volume with the treatment of one of CAIX inhibitors, N-[2-(2-methyl-5-nitro-imidazol-1-yl)ethyl]sulfamide, but the tumors needed only 14 days to reach the same volume with the treatment by vehicle alone. These inhibitors could specifically bind to the active site of CAIX and effectively suppress its activity. Radiolabeled inhibitors may be used for diagnostic or therapeutic purpose in CAIX-positive cancer cells.

Bao et al. have recently developed a quantitative in vivo optical imaging method for detection of CAIX as a marker of tumor hypoxia based on a near-infrared fluorescent derivative of the CAIX
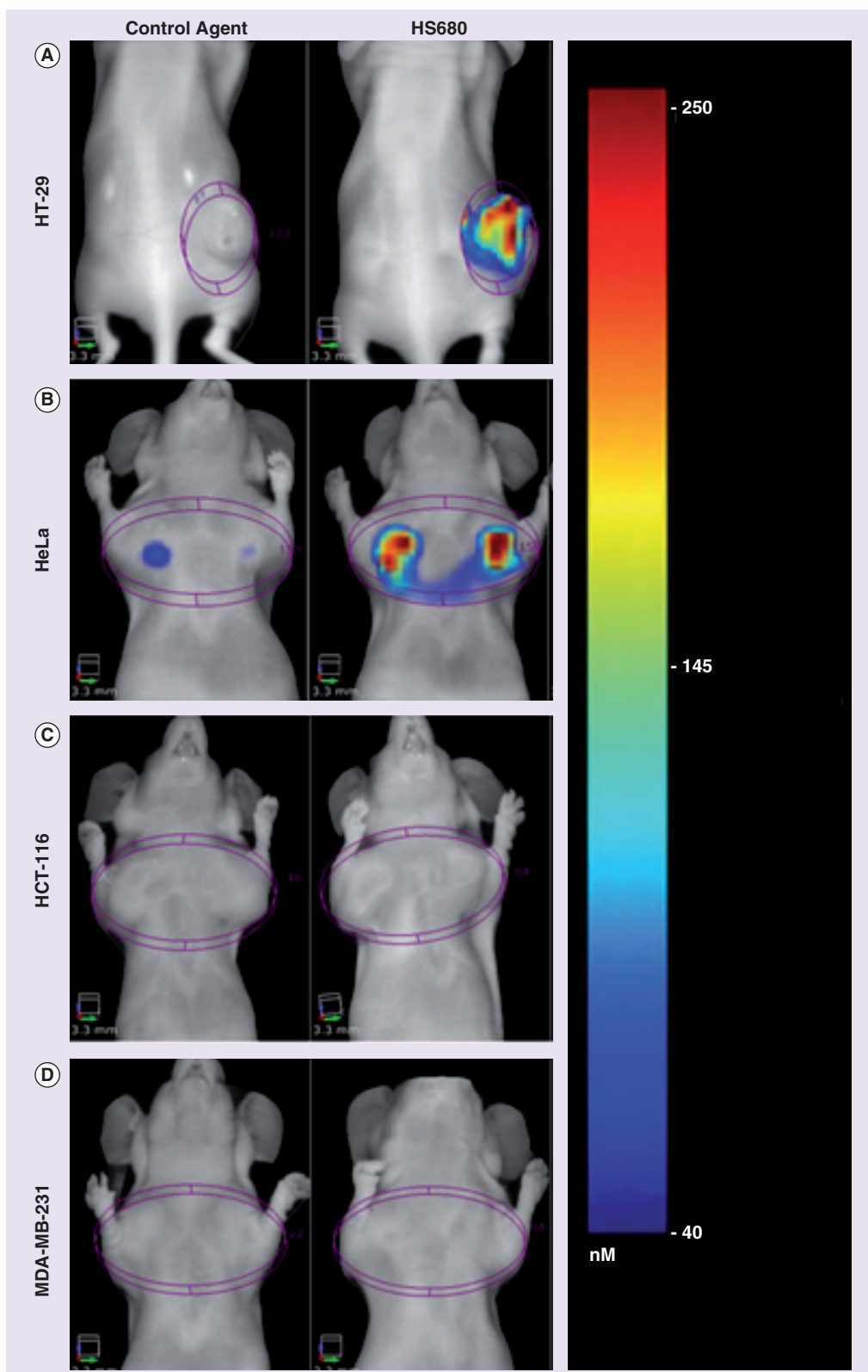
**Figure 2. *In vivo* FMT imaging of HS680 and control agent in mice bearing CAIX-positive (HT-29 and HeLa) and CAIX-negative (HCT-116 and MDA-MB-231) tumors.**
For color images please see online at www.futuremedicine.com/doi/full/10.2217/fon.15.11
Reproduced with permission from [32].

inhibitor acetazolamide referred as HS680 [32]. The authors demonstrated that CAIX negative cell lines HCT-116 and MDA-MB-231 xenogrfts showed low-HS680 fluorescent signal, whereas CAIX-positive cell lines such HT29 and HeLa xenografts had significant high fluorescent signal, and the author concluded that the potential of HS680 imaging to noninvasively quantify CAIX expression as a hypoxia biomarker, crucial to the study of the underlying biology of hypoxic tumors and the development and monitoring of novel anticancer therapies **(Figure 2)**. However, in the study, co-localization of HS680, pimonidazole and CAIX has been verified in HT29 tumor sections, but did not investigate in HCT-116 and

MDA-MB-231 xenografts which are assumed to present regions of hypoxia. However, results from this study indicates, to some extent, the cell line dependent feature of CAIX expression in cancer cells, and an example to show the limitation of the use of CAIX inhibitor for tumor hypoxia detection or cancer detection.

## CAIX-targeted imaging in clinical applications

Many sound results have been obtained in preclinical studies, some of which have been translated into clinical applications. cG250, a chimeric variant of G250 greatly diminishing the immunogenicity of the G250 antibody, has been
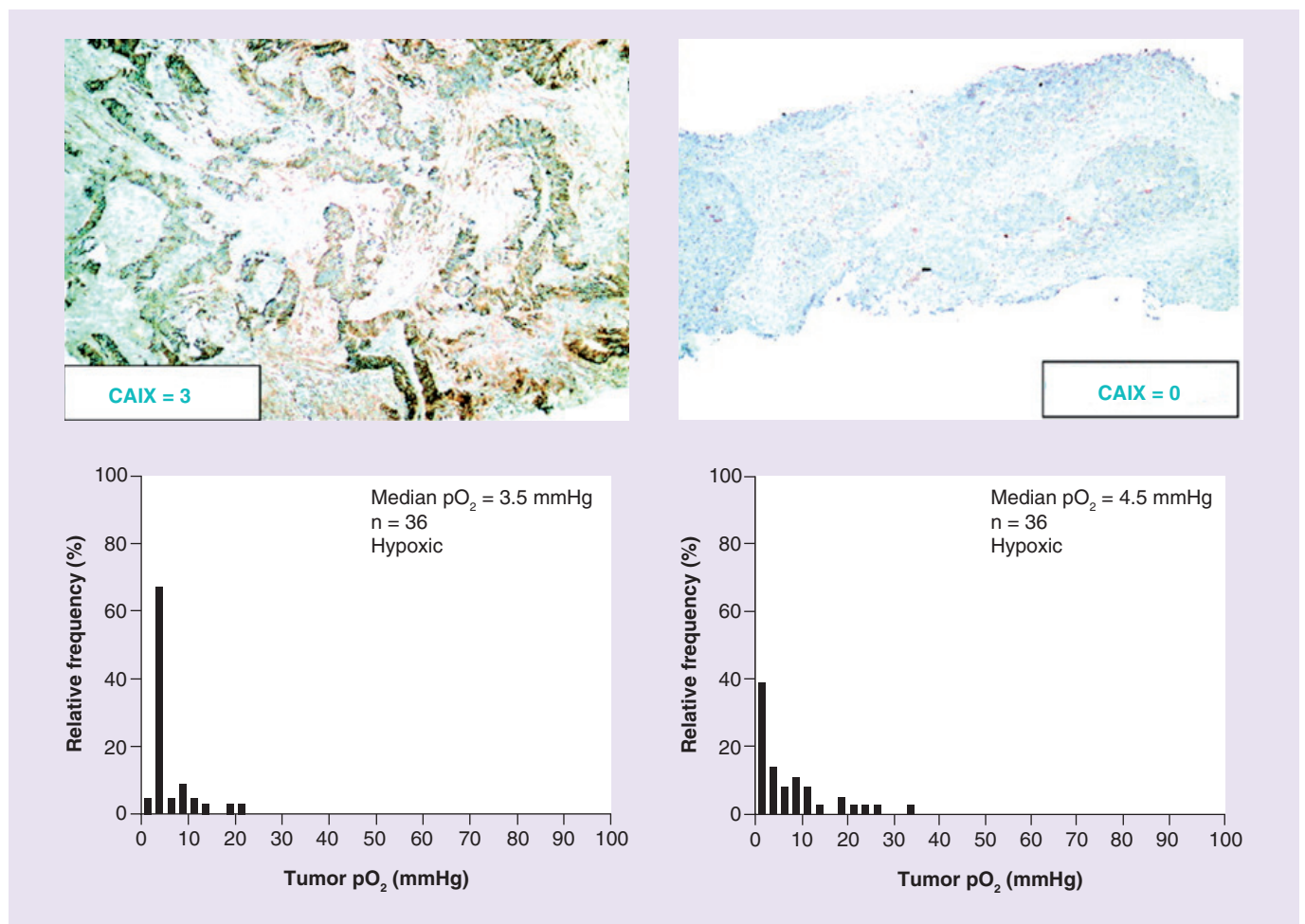


**Figure 3. Expression pattern and score of carbonic anhydrase IX with respective oxygen tension (pO$_2$).** There was strong expression of CAIX in a tumor section on the left upper figure, but there was almost no expression of CAIX in a tumor section shown on the right upper figure; both tumors had median pO$_2$ less than 10 mmHg (hypoxia). This figure was reproduced with permission from Mayer A, Höckel M, Vaupel P: Carbonic anhydrase IX expression and tumor oxygenation status do not correlate at the microregional level in locally advanced cancers of the uterine cervix.
CAIX: Carbonic anhydrase IX.
Reproduced with permission from [35] © by American Association for Cancer Research (2005).

used in clinical studies [42,67–68]. Pryma *et al.* conducted PET-CT scans in 26 patients with radiolabeled chimeric antibody [124]I-cG250 [69]. PET-CT imaging results indicated 15 patients were positive for [124]I-cG250 and ten were negative. For all tumors of surgical resection from [124]I-cG250 positive patients, the results of autoradiography were similar to the distribution of CAIX expression as showed by immunohistochemistry. [124]I-cG250 could be a good candidate for noninvasively imaging tumors with expression of CAIX. Genega *et al.* evaluated CAIX expression in 366 primary and metastatic renal neoplasms by immunohistochemical staining, they found that CAIX is more often expressed in clear cell RCC than other subtypes RCC (71
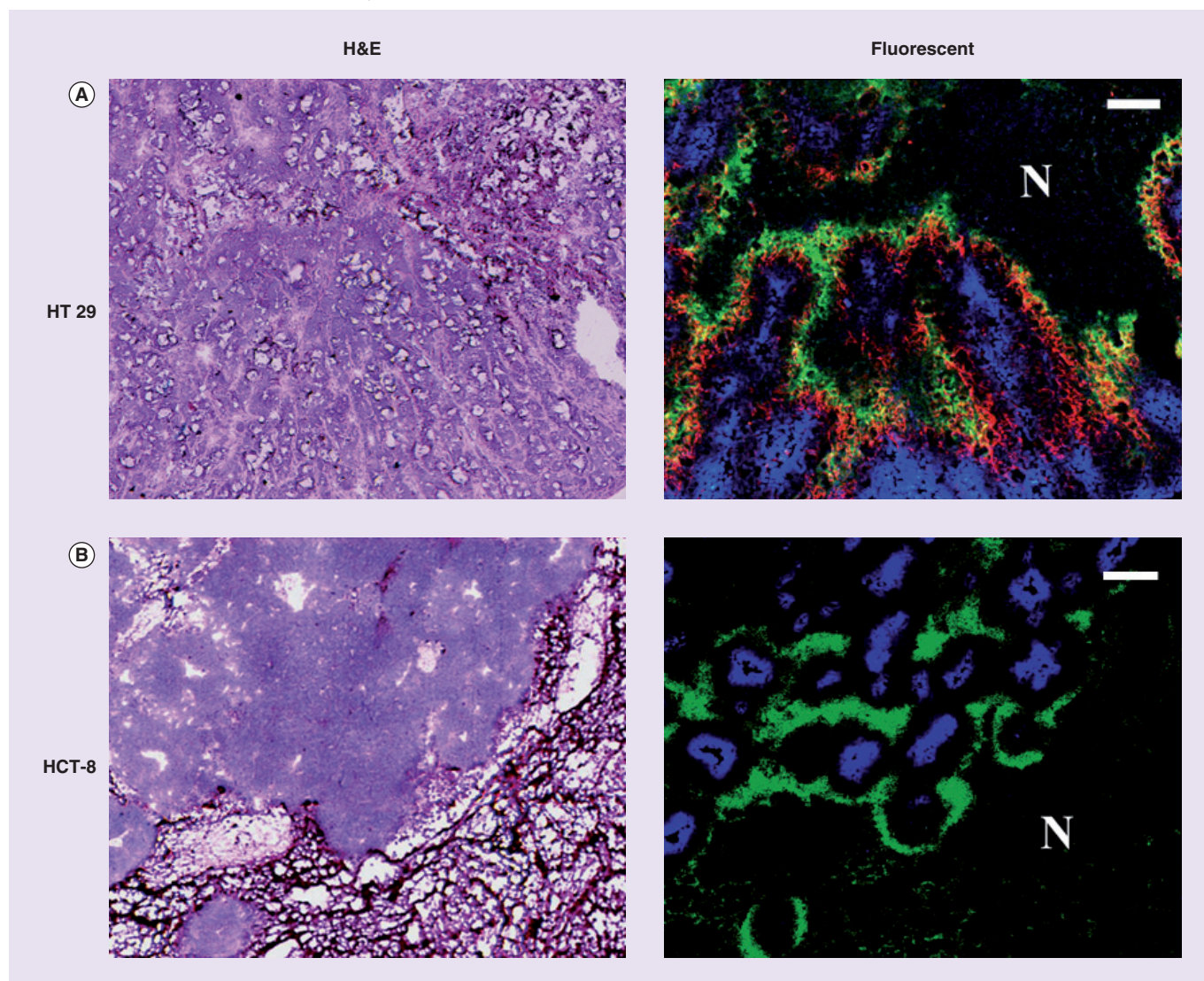


**Figure 4. Relationship between carbonic anhydrase IX, pimonidazole and Hoechst 33342 in HT29 and HCT-8 subcutaneous xenografts. (A)** H&E stained section from a macroscopic subcutaneous xenograft derived from the human colorectal carcinoma cell line HT29. Fluorescence overlay image (green = pimonidazole, red = carbonic anhydrase IX [CAIX], blue = Hoechst 33342) shows that there is broad concordance between pimonidazole and CAIX distributions. In particular all identifiable regions of enhanced pimonidazole binding have corresponding regions of enhanced CAIX expression. However, there are regions of CAIX positivity where pimonidazole binding is not seen. Scale bar, 200 μm. **(B)** H&E stained section from a macroscopic subcutaneous xenograft derived from the human colorectal carcinoma cell line HCT-8. Fluorescence overlay image (green = pimonidazole, blue = Hoechst 33342) of the identical section shown on the left. Similarly to the HT29 model, pimonidazole and Hoechst 33342 positivity appear mutually exclusive. However, CAIX is undetectable in HCT-8 tumor section where pimonidazole is stained positively. Scale bar: 200 μm. Reproduced with permission from [36].
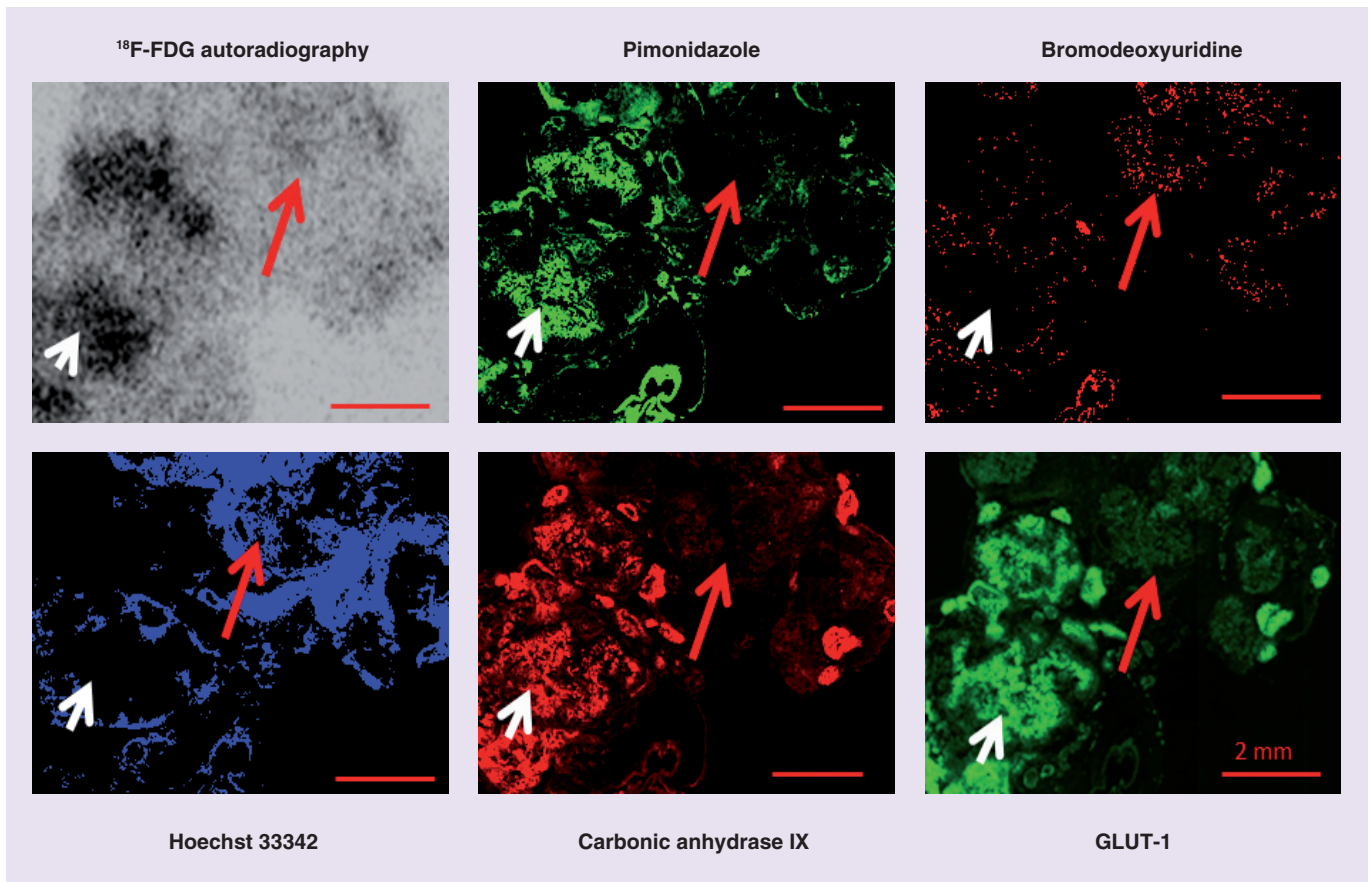
**Figure 5. Relationship between ¹⁸F-FDG uptake and hypoxia, proliferation and perfusion in HT29 serosal carcionomatosis hypoxic regions, where pimonidazole, GLUT-1 and carbonic anhydrase IX are stained positively, have high ¹⁸F-FDG uptake (white arrow).** Well perfused regions where Hoechst 33342 is positive and proliferating cancer cells are stained positively for bromodeoxyuridine have low ¹⁸F-FDG uptake (red arrow). All scale bars: 2 mm.
Reproduced with permission from [74].

vs 3%), accordingly, targeting CAIX imaging seems to be useful for distinguishing clear cell RCC from other RCC subtypes, but, apparently, it is unlikely to be a gold standard for RCC diagnosis [70].

## Limitation of CAIX-targeted imaging

In selected cancer cell lines, such as HT29 and HeLa cells, preclinical studies have documented that CAIX is a great target for cancer imaging and imaging tumor hypoxia. However, clinical studies indicate that CAIX is overexpressed in some cancer cells but not under conditions of hypoxia. CAIX is unlikely a good target for tumor hypoxia imaging and cancer detection.

Adams *et al.* analyzed 30,216 immunohistochemistry results from 117 articles and found there were only 35% expression rates of CAIX in human invasive breast cancer [71]. Mayer *et al.* found the presence of high CAIX expression in

hypoxic regions of some cancers but absence in the others **(Figure 3)** [35]. And we also documented that CAIX overexpression was similar to pimonidazole binding (exogenous hypoxic marker, specific when $pO_2$ <10 mmHg) in colon cancer HT29 xenografts growing in nude mice, while there was no immunohistochemical-staining-detectable CAIX expression in regions where pimonidazole was stained positively in the rectal cancer HCT-8 xenografts **(Figure 4)**.

Hendrickx *et al.* [72] conducted a therapeutic clinical trial on targeting CAIX of biliary cancer in three patients using ¹¹¹In-cG250 and ¹³¹I-cG250, respectively. Results from scintigrams of the abdomen of the patient either 5 days after injection of ¹¹¹In-cG250 or 5 days after injection of ¹³¹I-cG250, showed no apparent accumulation of radiolabeled cG250 in tumors. Immunohistochemical analyses revealed the expression of CAIX in tumor tissues of all three

patients (strong expression for two, moderate for one). So it is inappropriate that cG250, whether labeled with ^{131}I or ^{111}In, is used to target tumor tissues in patients bearing biliary cancer.

PET tracer 2-deoxy-2-[^{18}F]-fluoro-D-glucose (^{18}F-FDG) has better performance for imaging cancers, we and others had demonstrated that ^{18}F-FDG uptake was hypoxia-dependent **(Figure 5)** [21,73–76]. In addition, hypoxic portion of colorectal cancer HCT-8 xenografts had increased ^{18}F-FDG accumulation [21], but CAIX expression in hypoxic zones was undetectable by immunohistochemistry [36]. ^{18}F-FDG is much better PET tracer for cancer detection than targeting CAIX imaging strategies; approximately 95% of solid malignancies contain hypoxic cancer cells. The advantage of CAIX imaging strategies is that targeting CAIX imaging can be useful for screening purpose to provide information which patients are eligible for targeting CAIX therapy.

With respect to hypoxia imaging, there are few studies to compare CAIX targeted imaging strategies with ^{18}F-fluoromisonidazole (^{18}F-FMISO) PET, which may be an interesting project to further explore the limitation of CAIX targeted imaging. Although targeted

CAIX nuclear imaging has be documented, radiolabeled anti-CAIX antibodies autoradiography has compared with immunohistochemical visualization of CAIX expression, whereas a comparison with hypoxia marker pimonidazole (detecting cancer cells when pO2 <10 mmHg) immunohistochemical staining has been rarely performed.

## Conclusion

Although succeed in several types of cancer, such as in colorectal cancer HT29 xenografts growing in rodents, CAIX seems unlikely to serve as a universal target for cancer detection or tumor hypoxia visualization. The clinical use of antibodies, probes or inhibitors which targeted CAIX may be limited and unreliable for cancer and hypoxia visualization. However, imaging CAIX may be used to select candidates for anti-CAIX therapies.

## Future perspective

CAIX expression under hypoxic conditions is cell line dependent, targeted CAIX imaging may not be universal hypoxia imaging strategies, and, therefore, would be limited for clinical practice. However, CAIX overexpression may

## EXECUTIVE SUMMARY

### Background

- The presence of hypoxia is a general feature of most solid malignancies and hypoxia is considered as one of major factors for anticancer therapy failure.
- Carbonic anhydrase IX (CAIX) has been reported to be an endogenous hypoxia marker, CAIX monoclonal antibodies, their segments and inhibitors are developed for CAIX imaging.

### CAIX

- CAIX is a membrane-associated glycoprotein, consisting of an extracellular catalytic domain, a proteoglycan-like region (PG domain), a transmembrane anchor and a short C-terminal cytoplasmic tail.
- The overexpression of CAIX in normal human tissues is rare.
- CAIX regulates intracellular pH.

### CAIX & tumor hypoxia

- CAIX has been considered as an endogenous hypoxic marker.
- CAIX expression under hypoxic conditions may be dependent on cancer cell line or cancer type.
- CAIX may be unreliable as a universal target for cancer and tumor hypoxia visualization; CAIX imaging may identify patients that are likely to benefit from targeting CAIX therapy.

### Future perspective

- CAIX overexpression may play critical role on prediction anticancer therapeutic effect.
- The role of CAIX in addition to hypoxia in oncology should be addressed.

play a critical role in the prediction of anticancer therapeutic effect. CAIX overexpression is not universal in hypoxic cancer cells. Investigations on the outcome and progression CAIX-positive hypoxic cancer versus CAIX-negative hypoxic cancer should be conducted.

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