Carbonic anhydrase isozymes as diagnostic biomarkers and therapeutic targets

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Abstract

The early immunohistochemical studies of carbonic anhydrases (CAs) were mainly focused on their normal tissue distribution. Only a few studies included samples from pathologic diseases, particularly cancer. This line of research remained inactive until the discovery of CA IX – the first cancer-associated isozyme. The association of CA IX with hypoxic regions of tumors became obvious, and experimental results confirmed hypoxia regulation. CA IX is now widely considered a biomarker of tumor hypoxia and prognosis. Even though it has several characteristics of a promising biomarker, the implementation of CA IX in clinical pathology has progressed slowly. CA IX research has also produced promising therapeutic molecules, some of which are already in clinical trials. CA XII is another cancer-associated isozyme; however, it is not yet used as a clinical biomarker in routine diagnostics nor is it utilized in therapeutic applications. Surprisingly, the well-known isozyme CA II has turned out to be an attractive candidate as a diagnostic marker, at least in the special case of gastrointestinal stromal tumors.

As a conclusion, certain CA isozymes have definite promise as histopathological markers and therapeutic targets. Even though the implementation of new approaches is a slow process in clinical medicine, the first steps have been taken to utilize the unique properties of CA isozymes in diagnostics and therapy.

Keywords: Biomarker, Cancer, Carbonic anhydrase, Diagnostics, Hypoxia, Prognosis

Short introduction of mammalian carbonic anhydrases

Among vertebrates, the α-carbonic anhydrases (CAs) are the dominant enzyme forms, and they have distinctive subcellular localizations, tissue distributions, activities, and biological functions. Their key roles include various biological functions, such as the regulation of pH homeostasis, ion transport, gluconeogenesis, ureagenesis, respiration, bone resorption, as well as formation of

biological fluids. Thirteen active isozymes have been identified in mammals thus far: five cytoplasmic (CA I, CA II, CA III, CA VII, and CA XIII), five membrane-associated (CA IV, CA IX, CA XII, CA XIV, and CA XV), two mitochondrial (CA VA and CA VB), and one secreted form (CA VI) (Fig. 1) [1-4]. In addition, the CA gene family includes three carbonic anhydrase-related proteins (CARPs) that lack CA catalytic activity due to missing histidine residues in their active sites [5]. As with their catalytic activities, all isozymes also differ in their affinity for CA inhibitors. Some of the developed CA inhibitors are clinically used as therapeutic agents in the management of certain diseases, such as glaucoma and epilepsy [6-8]. It has become evident that CAs and their inhibitors offer interesting opportunities for both developing novel drugs and as diagnostic tools to improve the health and wellbeing of humans and other species.

CA II as a biomarker

CA II, although it is the most active and widely expressed isozyme in human tissues, has attracted much less attention in cancer research compared to the classical tumor-associated isozymes, CA IX and XII. It was first demonstrated by immunohistochemistry that colorectal adenomas and adenocarcinomas do not stain for CA II [9]. Low expression of CA II was later confirmed in a study where CA I, II and XIII were investigated in parallel tissue sections [10], as well as in a study where the expression of various CA isozymes was analyzed using a microarray technique [11].

CA II is highly expressed in the epithelium of the pancreatic ducts, where it is involved in the production of bicarbonate-rich pancreatic juice [2,12]. The expression rate of CA II in the ductal cells, which is sustained after malignant transformation, does not correlate with the malignancy of tumors, suggesting a limited value for CA II reactivity in histopathological diagnostics of pancreatic adenocarcinoma [12].

It is well-known that CA II is prominently present in both gastric parietal cells and bicarbonate-producing surface epithelial cells of the stomach, whereas it is only weakly expressed in the esophageal epithelium [13]. Recently, the expression and clinical significance of CA II, IX, and XII were investigated in Barrett's esophagus and esophageal adenocarcinoma (EAC) [14]. Even though CA II was significantly downregulated in metastatic disease, it was concluded that none of the tested isozymes could provide additional value as a biomarker for esophageal adenocarcinoma.

Two recent articles have provided congruent results showing that the reduction of CA II expression in gastric tumors is associated with tumor growth, metastasis, and poor prognosis. The first report demonstrated that the expression of CA II was significantly higher in the normal gastric mucosa than in the intraepithelial neoplasia and gastric carcinoma [15]. The positive signal for CA II was significantly more frequent in gastric carcinoma at early stages compared to more advanced stages. The rate of positive CA II signal was significantly lower in poorly differentiated gastric carcinoma than in moderately or well-differentiated tumors. The patients with CA II-positive tumors showed a better survival rate than those with CA II-negative tumors. The second publication confirmed that CA II expression is significantly decreased in gastric cancer compared with the normal gastric mucosa [16]. Low expression was significantly associated with tumor size, depth of invasion, lymph node involvement, distant metastasis and TNM stage, and it predicted poor survival in gastric cancer patients.

Viikilä and coworkers recently investigated the expression of CA II, VII, IX, and XII in a series of colorectal carcinoma samples from 593 patients [17]. Only CA II and XII showed statistically significant correlations to patient survival in that higher expression indicated poorer prognosis. CA II staining associated with the patient age group, while no other significant correlation was reported between the isozymes and various clinicopathological parameters.

The most promising results for CA II as a potential biomarker were published in 2010, when the enzyme was identified in gastrointestinal stromal tumors (GISTs) [18]. GISTs represent the most

common mesenchymal tumor category of the gastrointestinal tract and include tumors with low malignancy and those that behave as highly malignant metastasizing neoplasias. KIT expression is a nearly consistent phenotypic feature of GISTs, and oncogenic activation of KIT or PDGFRA receptor tyrosine kinase signaling is considered pathogenetically important [19]. GISTs originate from Cajal cells of the gastrointestinal wall [20]; thus, the tumors can arise in various gastrointestinal locations. Western blotting experiments first indicated that CA II is highly expressed in GIST cell lines [18]. Subsequently, CA II expression was analyzed in 175 GISTs, of which 95% showed positive immunostaining. The CA II expression in GISTs did not correlate with particular *KIT* or *PDGFRA* mutation types. CA II was absent or expressed at low levels in the other mesenchymal tumor categories that were analyzed in that particular study. High CA II expression was associated with a better disease-specific survival rate than low or no expression. The results indicated that CA II is quite selective to this tumor type among various mesenchymal tumors; therefore, it might be a useful biomarker in diagnostics.

Recently, CA II expression was studied in another type of gastrointestinal tumors, called pseudomyxoma peritonei [21]. Specimens were collected from 89 patients with this malignancy; the tissue sections were immunostained for CA II, and the expression was analyzed against the survival of the patients. Positive CA II expression was found in 65% of patients. The 5-year overall survival rates for the CA II-positive and -negative cases were 80% and 59%, respectively, reaching a clear statistical significance. It was suggested based on the results that the expression of CA II acts as an independent prognostic biomarker in pseudomyxoma peritonei.

CA II is expressed in at least many, if not in most, cases of hematological malignancies. Leppilampi and coworkers investigated the presence of CA isozymes in malignant hematopoietic cell lines and malignant blast cells of bone marrow samples [22]. Three out of six malignant hematopoietic cell lines expressed CA II, whereas no expression was detected for CA I, IX or XII. Positive reactions were found in 62% of acute myeloid leukemia samples, 73% of acute lymphoblastic leukemias, and

50% of chronic myelomonocytic leukemias. The results indicated that CA II expression is not restricted to one cell lineage but may result from a genetic aberration that occurs in both myeloid and lymphatic lineages or in their progenitor cells.

CA II is expressed in various brain tumors, including astrocytic tumors, oligodendrogliomas, ependymal and choroid plexus tumors, and tumors of nerve sheath cell origin [23]. In brain tumors, a significant fraction of CA II reactivity is, in fact, located in the capillary endothelium [24]. A similar pattern of ectopic CA II expression has been demonstrated in the endothelium of neovessels of several other cancers, including melanoma and esophageal, renal and lung tumors [25].

CA IX as a biomarker

Among different CA isozymes, CA IX has shown the highest promise as a potential biomarker of certain tumors. In fact, there are already over 1000 publications available on the combined topics of CA IX and cancer, although CA IX is not fully specific for cancer cells only. Among various normal tissues, CA IX is abundantly present in the basolateral plasma membranes of the gastrointestinal epithelia [26]. The highest levels of the enzyme are present in the epithelial cells of both gastric and gall bladder mucosa. The expression is prominent also in the proximal gut, i.e., in the duodenum and jejunum, and it is moderate in the ileum and colon, while it diminishes towards the rectum [27]. In contrast to the gastric epithelium, where the enzyme is highly expressed by the surface epithelial cells and parietal cells, the expression in the intestinal epithelium is confined to the deep proliferating areas, the crypts of Lieberkühn. As the cells migrate along the intestinal villus, they differentiate and gradually lose their CA IX expression, suggesting that it may have a role in the proliferation and differentiation of epithelial cells. In addition to the stomach, gallbladder and intestine, CA IX expression has been detected in the human biliary epithelium, pancreatic ducts, male reproductive organs and mesothelium [28-30,26,31].

CA IX is a highly active enzyme with catalytic activity in the same range with CA II [32]. The high enzyme activity of CA IX may be of potential significance for tumor growth and invasion. According to the current paradigm, CA IX functionally contributes to the acidification of the extracellular microenvironment surrounding cancer cells by interacting directly with ion transport proteins; accordingly, it may neutralize the intracellular space of tumor cells [33,34]. The protons produced by CA IX may remain outside and increase the acidity of the tumor microenvironment [35]. In fact, there are several pieces of evidence that CA IX physically and functionally interacts with bicarbonate transport proteins AE1, AE2, and AE3 and with sodium-dependent electroneutral bicarbonate cotransporter (NBCn1) [36-38]. CA IX also associates with proteins involved in amino acid transport, including AA transport heavy chain subunit, CD98hc (SLC3A2), L-type AA transporter, LAT1 (SLC7A5), alanine-serine-cysteine-preferring transporter 2, ASCT2 (SLC1A5), and sodium-coupled neutral amino acid transporter 2, SNAT2 (SLC38A2) [38].

It is well-documented that CA IX is highly expressed in various tumors, including those that arise from the GI tract [39,35]. As the first example, many colorectal tumors overexpress CA IX [40,11]. CA IX is the only CA isozyme that is overexpressed in hereditary nonpolyposis colorectal cancer (HNPCC) [11]. In colorectal tumors, CA IX has shown high expression in premalignant lesions and often a lower signal in poorly differentiated malignancies, suggesting that it might be a useful marker in the early diagnosis of colorectal tumorigenesis. More recent studies have indicated, however, that CA IX is not a prognostic factor in colorectal cancer [17].

Turner and coworkers were the first to suggest that tumor-associated CA IX may play a role in the proliferation and regeneration of esophageal squamous epithelium, and loss of its expression may be related to cancer progression in Barrett's-associated adenocarcinomas [41]. Later, it was reported that 44.5% of esophageal carcinomas show strong CA IX expression, and the high expression is an independent prognostic factor for shorter overall and disease-free survival [42]. In another study, high CA IX expression was associated with tumorigenesis markers BMI1, MCM4, and MCM7,

suggesting that CA IX may play a role in early tumorigenesis [43]. In contrast to the previous results, CA IX was not found to be a significant prognostic marker in patients with esophageal adenocarcinoma. In the most recent study, Nortunen and coworkers analyzed CA IX expression in a series of esophageal adenocarcinomas and Barrett´s esophagus [14]. The normal squamous epithelium of the esophagus was almost completely negative for CA IX. The signal was strong in gastric metaplasia and present in all cell types across the epithelium. The expression levels decreased from gastric metaplasia to intestinal metaplasia, dysplastic lesions, and finally to adenocarcinoma. In intestinal metaplasia, the expression was still strong and showed basal dominance. The CA IX expression in carcinomas showed no significant correlation with clinicopathological variables or survival, although high CA IX expression appeared to associate weakly with nodal spread.

In the liver, CA IX is localized to the biliary epithelial cells [26]. Analogously, CA IX is expressed by the biliary epithelial tumors [28]. It seems plausible that CA IX expression indicates better prognosis in intrahepatic cholangiocarcinoma [44], even though CA IX is typically associated with poor prognosis in many other tumor categories. One of these examples is hepatocellular carcinoma, in which CA IX is expressed only in a minority of cases. In a large cohort of patients, Kang and coworkers showed that 15.1% of the cases expressed CA IX at low level, and 5.2% belonged to the high CA IX-positive group [45]. CA IX expression was a prognostic factor for poorer survival after surgery for hepatocellular carcinoma. In addition, the high CA IX-positive group had even a poorer prognosis than the low CA IX-positive group. Recently, it was further confirmed that CA IX is, indeed, a predictive factor for poor prognosis after radical surgery for hepatocellular carcinoma [46]. Finkelmeier and coworkers recently published an interesting study where they analyzed circulating CA IX enzyme levels in patients with hepatocellular carcinoma or cirrhosis [47]. They included 215 patients with hepatocellular carcinoma in the study. The median serum CA IX concentration in patients with hepatocellular carcinoma was 370 pg/ml, and it was significantly

higher than in controls (41 pg/ml). The patients with high serum CA IX concentrations (>400 pg/ml) had an increased mortality risk. Surprisingly, the serum CA IX concentration in cirrhotic patients did not differ significantly from the patients with hepatocellular carcinoma. Higher CA IX levels in cirrhotic patients correlated with portal hypertension and esophageal varices, and the patients with ethanol-induced cirrhosis had the highest CA IX levels.

Ectopic expression through hypoxia regulation is an important hallmark of CA IX; therefore, CA IX is most highly expressed in tumors that originate from CA IX-negative tissues. Because the normal gastric mucosa contains the highest levels of CA IX among normal tissues, it was not surprising that gastric carcinomas showed relatively low expression [48]. Nakamura and coworkers have shown that the expression of CA IX in gastric cancer is predominantly regulated by the methylation of a single CpG rather than by hypoxia [49]. A subgroup of gastric cancers retains CA IX expression in cancer cells at the invasion front [50]. It has been shown that overexpression of CA IX is important for tumor invasion and metastasis; as such, it may serve as a useful prognostic indicator of long-term survival in patients with gastric adenocarcinoma [51].

CA IX is not prominently expressed in any normal neural tissues. Interestingly, it is highly expressed in some cases of brain tumors. The first publication on this topic was published by Haapasalo and coworkers, showing a positive signal for CA IX in 78% of astrocytomas [52]. The staining pattern followed the distribution of hypoxic regions within tumor specimens. The CA IX immunoreactivity showed a strong association with tumor malignancy grades. CA IX showed no association with p53 expression, nor did it correlate with epidermal growth factor receptor-amplification, apoptosis, or cell proliferation. CA IX intensity had significant prognostic value in survival analysis, which was confirmed later by other researchers [53]. In oligodendroglial tumors, CA IX was positive in 80% of the cases, and it again correlated with poorer outcome [54]. CA IX expression has also been studied in rare pediatric brain tumors, including primitive neuroectodermal tumors and medulloblastomas [55]. CA IX-positive staining was observed in 23% of the cases, and

its expression predicted poor prognosis of patients. Since the presence of hypoxic and necrotic areas represents an important criterion in glioma diagnostics, CA IX has become a useful biomarker for this tumor category. Accordingly, the neuropathologists of Tampere University Hospital already utilize CA IX immunostaining as a routine diagnostic asset for this clinical purpose (Dr. Hannu Haapasalo, personal communication).

CA IX expression has been studied in a variety of lung cancers. Ramsey and coworkers analyzed CA IX immunohistochemical expression in pulmonary/pleural tumors, including metastatic clear cell renal carcinoma of the lung, mesothelioma, squamous cell carcinoma, small cell carcinoma, and adenocarcinoma [56]. All cases of metastatic clear cell carcinomas and mesotheliomas were positive for CA IX. Most cases of lung squamous cell carcinoma and small cell carcinoma were positive, while the enzyme was less frequently present in pulmonary adenocarcinoma. The high expression of CA IX in mesotheliomas was later confirmed in a series of 27 malignant pleural mesotheliomas [57]. According to the immunohistochemical and follow-up data, CA IX expression predicts a poor survival rate in lung cancer [58,59]. The presence of CA IX has been specifically linked to the expression of proteins that are involved in angiogenesis, apoptosis inhibition, and cell-cell adhesion disruption, which explains the strong association of the enzyme with poor clinical outcomes [60]. However, recent studies have indicated that the inclusion of CA IX as one criterion does not improve the prognostic accuracy of blood biomarkers for the diagnostics of non-small cell lung cancer [61].

Cervical cancer was one of the first cancer types in which CA IX expression was studied in detail [62]. Loncaster *et al.* (2001) showed clinical evidence that CA IX expression in cervical cancer correlates with the levels of tumor hypoxia and associates with a poor prognosis of the disease [63]. The authors suggested that the level of CA IX expression may be used to select patients who would benefit most from hypoxia-modification therapies or bioreductive drugs. Maseide and colleagues noticed that high CA IX expression predicts a poor prognosis for patients with soft tissue sarcoma

[64]. Hynninen and coworkers investigated the expression of CA II, CA IX, and CA XII in a series of gynecological malignancies, including adenocarcinomas and mesenchymal tumors, such as sarcomas and leiomyomas [65]. Positive staining of CA II, CA IX, and CA XII was detected in many cases of sarcomas. The study confirmed the earlier positive results of CA IX expression in leiomyosarcoma [66], and it also added new data on the expression levels of CA IX in stromal sarcomas and mixed Müllerian tumors. In addition to the results for CA IX, the findings showed that CA II and CA XII are often weakly or moderately expressed in these mesenchymal tumors. Among these tumors, all isozymes showed variable staining results, suggesting that they have only limited value in sarcoma diagnostics if used alone. The specimens involved a total of 33 leiomyomas, all of which were negative for CA II and CA XII. Previously, using another series of tumors, Mayer and his coworkers reported that all leiomyomas are negative for CA IX [66]. In contrast, Hynninen and colleagues reported that 33 leiomyoma specimens included five cases that were CA IX-positive. The biological role and mechanism of CA IX induction in leiomyomas remained unresolved, however. There were at least no other visible signs of hypoxia in the tissue sections.

Ovarian tumors is another category of tumors in which CA IX is highly expressed [67]. Most cases of borderline mucinous cystadenomas, mucinous cystadenocarcinomas and serous cystadenocarcinomas are moderately or strongly positive for CA IX. In malignant tumors, the expression patterns have shown clear correlations to hypoxic regions. The high expression levels of CA IX in mucinous and serous cystadenocarcinomas suggested that these tumors could be considered potential candidates for CA IX-targeted therapy.

The current literature already includes a number of publications on CA IX expression in breast tumors. A study by Bartosová and colleagues indicated that ectopic activation of the *CA9* gene may be implicated in breast carcinogenesis, and it also suggested that CA IX could be a breast cancer marker [68]. The main conclusion from several expression studies is that CA IX indicates poor

prognosis in breast cancer [69-71], even though Span and colleagues demonstrated that CA IX is more predictive than prognostic in this cancer type [72].

The expression of CA IX has been examined in head and neck squamous cell carcinoma (HNSCC) [73]. The enzyme was related to the location of tumor microvessels, angiogenesis, necrosis, and tumor stage, and it was considered a potential target for future therapy in HNSCC. Follow-up studies have included CA IX in the panels of possible predictive markers in HNSCC [74,75]. Although combinations of markers have been associated with treatment outcome, their clinical value as predictive factors must still be established [76].

Both CA IX and CA XII have been investigated in both the normal skin and skin tumors [77]. In the normal skin, the highest expression of CA IX was detected in hair follicles, sebaceous glands, and basal parts of the epidermis. CA XII was detected in all epithelial components of the skin. Both CA IX and CA XII expression levels were significantly different in epidermal, appendageal, and melanocytic tumor categories. Both CA IX and XII showed the most intense immunostaining in epidermal tumors, whereas virtually all melanocytic tumors were devoid of CA IX and XII immunostaining. In premalignant lesions, CA IX expression significantly increased when the tumors progressed to more severe dysplasia forms.

The story about CA IX in kidney tumors and its role as a prognostic marker is rather complicated because of an alternative mechanism of upregulation of CA IX expression due to von Hippel-Lindau gene mutations in certain tumors. Human renal cancer cell lines and renal cancers have shown high expression of CA IX mRNA and CA IX protein [78,79]. CA IX is mostly, but not fully, specific to clear cell renal cell carcinoma among various kidney tumors. Notably, the most common tumor of the pediatric kidney, Wilms' tumor, has shown 63% positivity for CA IX, although the median fraction of positive cells was only 5% [80]. Sandlund and coworkers assessed CA IX expression in different subtypes of renal cell cancer [81]. They found that the expression is higher in conventional clear cell renal cell carcinoma compared to other renal cancer types. They also

reported that the patients with clear cell carcinoma have a less favorable prognosis when the CA IX expression is low. In line with those findings, CA IX has also been described as a prognostic marker in metastatic clear cell carcinoma [82]. Limited CA IX expression in the area of 85% or less has been associated with poorer cancer-specific survival [83]. This exact criterion seems to be valid in metastatic tumors only, and based on present knowledge, CA IX expression may not represent a reliable prognostic factor in localized clear cell renal cell carcinomas [83]. The present literature available on CA IX in renal cancer suggests that the enzyme may represent not only a useful prognostic marker for metastatic clear cell renal cell carcinoma but also a promising therapeutic target for novel oncological applications, including immunotherapy and radioisotopic methods [84,35].

Thyroid cancers express both CA IX and CA XII with different patterns [85]. Among various thyroid tumors, the highest expression of CA IX was reported in medullary thyroid carcinoma and anaplastic carcinoma, where positive immunostaining was reported in 91.67% and 100% of cases, respectively. Only limited expression of CA IX was detected in well-differentiated tumors (3.8% of papillary thyroid carcinoma and 12% of follicular thyroid carcinoma). The highest positivity for CA XII was found in papillary carcinoma (91.7%).

Very recent investigations have demonstrated CA IX expression in lymphoma cells [86]. More specifically, positive signal was found in Reed-Sternberg cells of Hodgkin´s lymphoma in 39/81 samples (48.1%). In contrast, CA XII expression in these cells was present in only 18/77 samples (23.4%). For the CA IX-positive group, 72 month-progression free survival was significantly lower compared with the CA IX-negative cases, while the overall survival did not differ significantly.

As this chapter indicates, there have been many studies investigating the prognostic value of CA IX expression in patients with solid tumors. In 2016, van Kuijk and colleagues published the first meta-analysis covering 147 studies on CA IX expression in various tumor categories (excluding renal cancer because of different regulation) [87]. Overall, the results showed that high CA IX expression

is an adverse prognostic marker in solid tumors (excluding renal tumors). A strong association between high expression and poor prognosis was reported in the majority of different tumor sites, supporting a pivotal role of CA IX in disease progression and treatment resistance in various cancers.

CA IX immunoassay data

Currently, CA IX immunoassay reagents and methods are available from several commercial sources, even though it is not always possible to define the original manufacturer based on the incomplete datasheet or other public data. Some examples of the commercial assays are shown in Table 1. Many articles have been already published where CA IX concentrations were detected from human biological fluids, including serum, plasma, pleural effusion, or urine. In most studies, the data have been collected from patients with renal cancer [88-94]. Based on immunohistochemical data, it is obvious that CA IX expression is much more common in clear cell renal cell carcinoma than in other neoplasms of the kidney [95]. Immunoassay results of CA IX have recently shown that the expression levels are very high in the plasma of patients with clear cell renal cell carcinoma [88]. High serum CA IX levels are significantly associated with shorter overall survival [89], suggesting that increased shedding of the enzyme into circulation is a hallmark for poor prognosis.

Woelber and colleagues analyzed the CA IX levels in both ovarian [96] and cervical cancer patients [97]. They found that the enzyme concentration did not change significantly during the first-line therapy of ovarian cancer and were not prognostically relevant. Similarly, the serum concentrations of CA IX did not correlate with intratumoral expression of the enzyme or other clinicopathological variables in cervical cancer. Ilie and colleagues analyzed CA IX levels from patients with non-small cell lung cancer and found that the high plasma level of CA IX is an independent biomarker of poor prognosis [98]. Ostheimer and coworkers combined three markers, including osteopontin, vascular

endothelial growth factor and CA IX [99]. They found that high pretreatment plasma levels of these markers additively correlated with prognosis in M0-stage non-small cell lung cancer. CA IX concentrations have also been studied from pleural effusions of patients with various pathologies [100]. It was found that CA IX levels were significantly higher in the effusions collected from the patients with malignant diseases compared to those collected from patients with various benign diseases. Rosenberg and colleagues investigated CA IX levels by ELISA in patients with head and neck cancer [101]. They found that high pretreatment CA IX concentration is a negative prognostic factor in locally advanced tumors.

Table 1. Examples of commercially available ELISA immunoassays for CA IX.

Manufacturer/Distributor	Name of the assay	Detection range	References
Boster Biological Technology	PicoKine™ ELISA	15.6 pg/ml – 1000 pg/ml	
R&D Systems	Human Carbonic Anhydrase IX Quantikine ELISA Kit	15.6-1000 pg/ml	[102-104,89]
R&D Systems	Human Carbonic Anhydrase IX DuoSet ELISA	15.6-1000 pg/ml	[80,100]
Biomatik	Carbonic Anhydrase IX (Human) ELISA Kit	32-2000 pg/ml	
LifeSpan BioSciences	Human CA9 / Carbonic Anhydrase IX ELISA Kit (CLIA)	8-500 pg/ml	
LifeSpan BioSciences	Human CA9 / Carbonic Anhydrase IX ELISA Kit (Sandwich ELISA)	0.205-20 pg/ml	
LifeSpan BioSciences	Human CA9 / Carbonic Anhydrase IX ELISA Kit (Sandwich ELISA)	32-2000 pg/ml	
LifeSpan BioSciences	Human CA9 / Carbonic Anhydrase IX ELISA Kit (Sandwich ELISA)	15.6-1000 pg/ml	
Assay Solution	Human Carbonic Anhydrase IX ELISA Kit (Colorimetric)	31.3-2000 pg/ml	
GenWay Biotech	Human Carbonic Anhydrase IX ELISA Kit	32-2000 pg/ml	

Aviva Systems Biology	Carbonic Anhydrase IX ELISA Kit (Human)	32-2000 pg/ml
Biorbyt	Human Carbonic Anhydrase IX (CA9) ELISA kit	-
Bioassay Technology Laboratory	Human Carbonic Anhydrase 9 ELISA kit	50-30000 pg/ml
Creative Diagnostics	Carbonic Anhydrase IX ELISA Kit	32-2000 pg/ml
Pacific Biomarkers	CA 9 (Carbonic Anhydrase IX)	-
Sigma-Aldrich	Human Carbonic Anhydrase IX ELISA Kit	-
Cloud-Clone Corp.	ELISA Kit for Carbonic Anhydrase IX (CA9)	7.81-500 pg/ml

CA XII in cancer

CA XII is broadly similar in overall structure to CA IX, excluding the proteoglycan-like domain of CA IX. The expression of CA XII is also induced by hypoxic conditions [33], but its distribution in tissues does not correlate with hypoxic regions to the same extent as CA IX. It has been demonstrated that the expression of CA XII is under estrogen receptor regulation, and the expression in breast tumors is associated with positive estrogen alpha receptor status [105-107].

Like CA IX, CA XII may also function in metabolons together with ion transport proteins. It has been reported that it regulates the function of the chloride-bicarbonate exchanger (AE2) [108,109]. Mutations in the *CA12* gene have been associated with an autosomal recessive form of salt wasting disease that results in hyponatremia in some Bedouin families [110,111]. Clinical symptoms of the trait include high sweat chloride concentration, dehydration, and failure to thrive in infancy.

Expression of CA XII has been quite extensively studied in both normal tissues and several types of cancer. It is expressed in the normal kidney [79], colon [112], endometrium [113], and skin [77], and its heterogenous expression pattern in tumors may reduce its potential as a biomarker [35]. Expression of CA XII transcripts in nonpigmented epithelial cells of retina has also been reported [114], and these epithelial cells from glaucoma patients showed increased *CA12* gene expression.

From these results, it has been concluded that CA XII is expressed in ciliary cells, and thus, may be involved in aqueous humor production.

One of the first publications on CA XII demonstrated its distribution in colorectal tumors [112]. Most cases of adenomatous polyps were positive for CA XII, and the staining became more diffusely spread within the lesion with a more severe grade of dysplasia. Among 20 malignant colorectal tumors, 19 showed positive reactions that were typically diffuse, being present in both the superficial and deep parts of the mucosa. In contrast, the normal colorectal mucosa shows CA XII-positive signal in the superficial part of the mucosa, whereas staining is usually absent in the deep part of the mucosa. Another early publication described the expression of CA XII in various tumor categories [31]. The highest rates of distinct positive signal were reported in cervical carcinomas and intraepithelial neoplasias, endometrial carcinoma, ovarian carcinomas and cystadenomas, breast ductal and lobular carcinomas, renal cancers excluding Wilms' tumor, colon adenomas, and gliomas.

The original discovery of CA XII was published almost simultaneously by two independent groups [115,33]. The first findings already linked CA XII to kidney function and renal cancer. In the human kidney, CA XII was located to the basolateral plasma membrane of the epithelial cells in the thick ascending limb of Henle, distal convoluted tubules, and collecting ducts [79]. A weak basolateral signal was also detected in the epithelium of the proximal convoluted tubules. In a series of 31 renal tumors, the enzyme showed moderate or strong expression in most oncocytomas and clear cell carcinomas.

CA XII seems to be expressed in the normal stratified squamous epithelium, including the esophagus and skin [77,14]. In pathological tumor stage 2-3 esophageal squamous cell carcinoma, the 3-year survival rate of patients with the high-grade expression of CA XII (29.1%) was significantly lower than that of patients with the low-grade expression of CA XII (70.3%) [116]. A

multivariate analysis showed that the expression of CA XII was one of the most important independent prognostic factors following radical esophagectomy in tumor stage 3-4 carcinomas.

An alternatively spliced form of CA XII is expressed in brain tumors [117]. RT-PCR revealed that the enzyme present in diffuse astrocytomas is mainly encoded by a shorter mRNA variant. Anti-CA XII antibody recognized both isoforms in the glioblastoma cell lines. Most diffusely infiltrating astrocytomas (98%) showed positive immunoreactions for CA XII protein. Importantly, the expression correlated with poorer patient prognosis in univariate and multivariate survival analyses. The absence of 11 amino acids in the short variant, which seems to be a common form in astrocytomas, may affect the normal quaternary structure and biological function of CA XII.

To date, there have been a few studies elucidating the prognostic value of CA XII. Watson and colleagues examined the CA XII expression in a series of 103 cases of invasive breast cancer and found a positive correlation with a lower relapse rate and a better survival [105]. Kim and coworkers analyzed both CA IX and XII expression in cervical cancer and found CA IX and CA XII transcript expression in 62.7% and 88.1% of tumors, respectively [118]. Multivariate analysis revealed that CA IX expression was the most significant factor associated with lower metastasis-free survival, whereas CA XII expression was linked to a lower risk of metastasis and better survival.

By comparing the current literature on CA IX and XII, it is obvious that CA IX has greater promise as a histological marker protein. Nevertheless, CA XII is physiologically an interesting member of the CA family, and its exact roles still deserve further investigations.

Carbonic anhydrases as therapeutic targets

CA research has produced large amounts of data on the distribution, functions and clinical relevance of different isozymes. These enzymes have been considered therapeutic targets for

treatments of various diseases, such as glaucoma, mountain sickness, brain edema, cancer, and epilepsy. In most cases, CA II has been considered the main target enzyme, even though several isozymes are typically expressed in target tissues and inhibitors can inhibit several isozymes with different affinities. Several drugs with CA inhibitory properties are clinically used, and more are under development to treat important diseases. The clinically approved drugs with CA inhibition properties include acetazolamide, methazolamide, ethoxzolamide, dichlorphenamide, dorzolamide, brinzolamide, topiramate, zonisamide, lacosamide, imatinib, and statins [39,119-121]. Because other chapters of this book describe different clinical targets in great detail, only glaucoma and cancer are briefly mentioned here as examples.

Glaucoma has been a major disease target where CA inhibition has been proven a useful therapeutic option. In the 1950's, the effect of acetazolamide on intraocular pressure was first demonstrated [122]. The first generation CA inhibitors for treatment of glaucoma included systematically acting drugs: acetazolamide, methazolamide, and dichlorphenamide [123]. The second generation CA inhibitors were topically acting sulfonamides, dorzolamide and brinzolamide, which possess both water solubility and liposolubility to penetrate the cornea, and they can enter the ciliary process where the CAs are present. More recently, the third generation CA sulfonamide inhibitors have been developed based on the novel "tail approach". These topically administered compounds have shown 2-3 times higher effects in reducing intraocular pressure compared to dorzolamide [123]. Supuran's group has suggested that dithiocarbamates could represent the fourth CA inhibitor group for antiglaucoma therapy [123,124]. Many dithiocarbamates inhibit CA II in the low nanomolar range, are easy to synthesize, and possess excellent water solubility. Therefore, some dithiocarbamates may arrive in clinical testing soon.

The connections of CAs with various forms of cancer are obvious; therefore, at least CA IX and CA XII are considered potential targets for cancer therapy. Supuran's and DeSimone's groups have recently published an extensive review article covering CA IX as a potential therapeutic target

enzyme in primary tumors, metastases, and cancer stem cells [125]. Their review describes state-of-the-art of studies on CA IX including structural, functional, and biomedical aspects, as well as the development of molecules with diagnostic and therapeutic potential. Crystal structures of both CA IX and CA XII have been previously described [126,127]. The knowledge of the three dimensional structures of both enzymes has made the design of selective inhibitors more straightforward. There are also several pieces of promising results showing that inactivation of CA IX and/or CA XII indeed inhibits the growth or invasion capacity of cancer cells [128-131]. Combination therapies using CA inhibitors together with other anticancer drugs may represent novel options for improved treatment efficiency, especially in hard-to-treat cancers [132].

Concluding remarks

During the last two decades, a great number of publications have suggested CA isozymes as potential biomarkers and therapeutic targets. Most of the studies have illustrated CA expression in various tumors, and many suggested CA isozymes – mainly CA IX and XII – as prognostic factors. In addition, immunological assays have been recently developed in order to monitor CA IX levels in biological samples. The observed association between cancers and different CA isozymes has already stimulated translational CA research, which will hopefully lead to attractive novel discoveries that will provide new hope for cancer patients.

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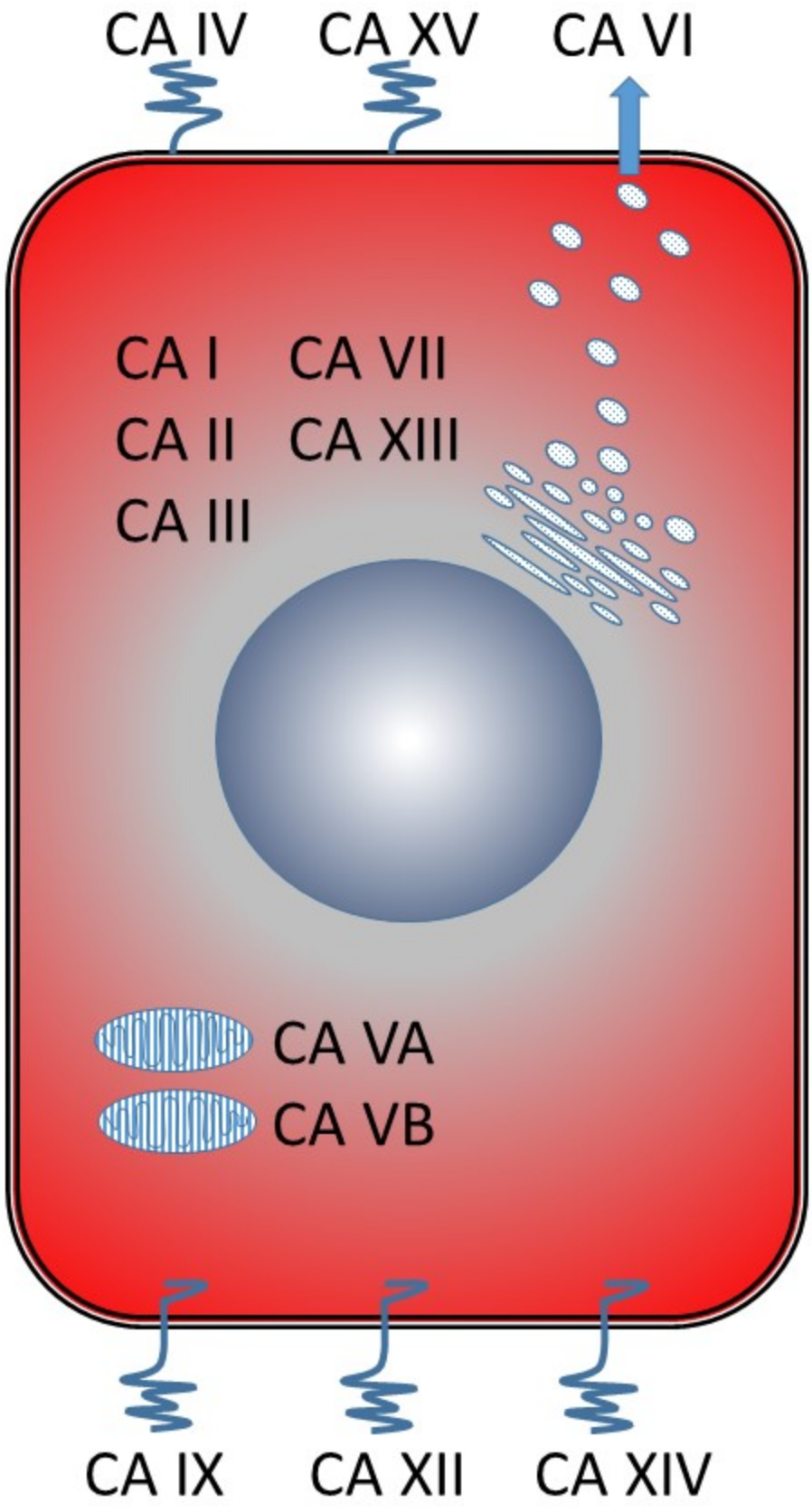
Figure legend

Figure 1. A typical subcellular localization pattern of the enzymatically active mammalian carbonic anhydrases. CA IV and CA XV are anchored to the plasma membrane through a glycosyl phosphatidylinositol (GPI) linkage. CA XV is not expressed in human tissues [4]. CA IX, XII and XIV are transmembrane enzymes whose catalytic sites are located to the cell exterior. CA IX and XII are confined to the basolateral plasma membrane, whereas CA XIV has also shown expression at the apical membrane [133]. CA VA and VB are mitochondrial enzymes, and CA VI is secreted via secretory granules into milk and saliva. The remaining isozymes are expressed in the cytosol.


CA IV
CA XV
CA VI
CA I
CA II
CA III
CA VII
CA XIII
CA VA
CA VB
CA IX
CA XII
CA XIV