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Carbonic Anhydrase As Biomarker For Cancer Diagnosis

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Abstract *—Carbonic Anhydrase (CA) is an enzyme that is integrated into the cell membrane that stimulates the reversible hydration of CO2 producing bicarbonate and hydrogen ions, and its study has gained relevance in the last years because it is related to several types of cancer such as; lung, kidney, colon, uterus, and stomach cancer (4, 5, 6,7,8). Therefore, different studies report carbonic anhydrase as an excellent biomarker for the early detection of lung cancer.*

Over the last few years, it is being used as a biomarker for the detection of lung cancer, as mentioned by Lin et al. (48), in their work, where they report a direct relationship between the overexpression of carbonic anhydrase and lung cancer. The present study assesses different methods where techniques are used to evaluate it by means of an electrochemical biosensor. These techniques will enable us to continue with the analysis of the characteristics that allow the recognition of carbon anhydrase in a range of 1.08-1.32mM with an acceptable sensitivity with a response time of 1s, demonstrating a promising technology for the application of lung cancer detection.

Keywords — *Carbonic Anhydrase, lung Cancer, diagnostics, biomarker.*

I. INTRODUCTION

Carbonic Anhydrase (CA) or carbonate dehydratases is an enzyme integrated into the cell membrane that catalyzes the reversible hydration of CO2 by producing bicarbonate and hydrogen ions. The catalytic activity allows the intracellular pH to be maintained in a range conducive to the survival of cancer cells (1).

Its catalytic activity is closely related to its conical structure and the presence of a metal ion, proper of metalloproteins (2), it has been found 16 isoforms that share a similar 3D structure (CA I, II, III, IV, VA, VB, VI, VII, VIII, IX, X, XI, XII, XIII, XIV and XV) distributed in different species (3).

Among all the isoforms, the carbonic anhydrase IX has gained relevance in recent years because it is related to several types of cancer such as lung cancer, kidney, colon, uterus, and stomach (4, 5, 6, 7, and 8). Therefore, different studies report carbonic anhydrase as an excellent biomarker to detect lung cancer early.

In this article, we make a bibliographic review describing the structure, function, bioinformatics studies, utilization as a biomarker, and new technology for the diagnosis.

II. STRUCTURE AND ISOFORMS

Discovered in the erythrocyte and described for the first time in 1933 (9), the Carbonic Anhydrase (CA) has been widely studied because of its close relationship with different pathologies due to its broad distribution in human tissues; furthermore, it has been attributed an important characteristic as a mediator in the pH of tumor cells allowing their survival and proliferation by modulating bicarbonate and proton concentrations (10).

CA is a group of metalloproteins that appear in the 3 forms of life (animals, plants and microorganisms) categorized in 6 different classes (α, β, γ, δ, ζ and η) (3) that despite having different amino acid sequence and three-dimensional structure catalyze the reversible reaction of hydration of carbon dioxide and bicarbonate in an identical way (9,11). Zn+2 ion coordinated by 3 histidine residues and a water molecule (H2O) are responsible for forming the active site of CA. Such reaction can be divided into three steps: in the first step, deprotonation of the water molecule attached to the Zn+2 ion releases a proton, leaving a hydroxide attached to the Zn+2 ion, which reacts with the carbonyl of the CO2 to form a molecule of HCO3− attached to Zn+2 (achieving the second step); lastly, HCO3− is replaced by a water molecule regaining its original active site (12, 13).

From the 6 classes mentioned, CA-α is the best characterized, and it is mainly found in vertebrates (14). It is divided into 4 groups according to their cellular and subcellular location, that is, cytosolic, mitochondrial, secretory, and membrane-associated. Likewise, CA-α can be divided into 16 isoforms that share a similar 3D structure (CA I, II, III, IV, VA, VB, VI, VII, VIII, IX, X, XI, XII, XIII, XIV y XV); however, they differ in amino acid sequence, enzymatic activity and degree of inhibition with CA inhibitors (9,12,16,17).

From a structural standpoint, most CA-α are monomeric, although homodimers have also been reported in some human and bacterial enzymes. Taking as an example the CA II isoform (Fig. 1A), it has a characteristic shape that is ovoid type presenting 10 central antiparallel betas sheets

surrounded by several propellers and additional betas sheets. The active site (Fig. 1B) is located in a large cone-shaped cavity that reaches the center of the enzyme, where we can found Zn+2 ion in coordination with preserved His residues (His 94, His 96, and His 119) and a water/hydroxide molecule (10). A particular characteristic of the amino acids present in the active site of the CA II isoform is that half of them are hydrophobic and the other half hydrophilic, thus subdividing the active site into two sub-sites capable of interacting with different classes of inhibitors. On the other hand, CA-β is catalytically active as dimers or tetramers or as multiples of these (e.g., hexamers or octamers), especially in plant enzymes. Finally, CA-γ is catalytically active as a trimer with 3 active sites containing the Zn+2 ion located at the interface of 2 monomers (10, 11).

In the human being, there are only 15 codified isoforms, 12 (CA I, II, III, IV, VA, VB, VI, VII, IX, XII, XIII, XIV) coordinate with a zinc molecule in the active site, and 3 of them (CA VIII, X, and XI) lack this metal, or they're His residues are substituted, known as AC-related proteins (CARPs) (2,9).

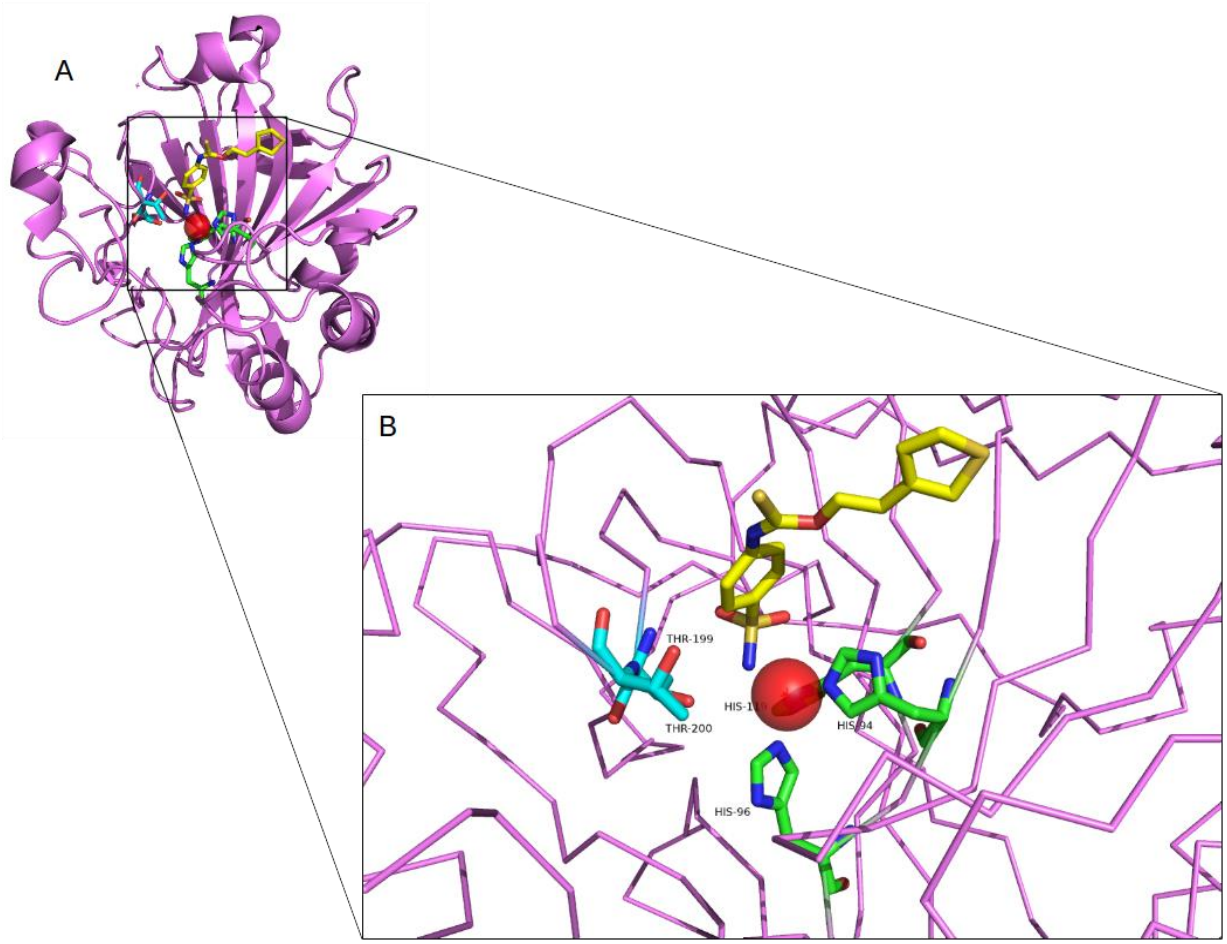

Fig 1: A) Carbonic Anhydrase structure represented in the cartoon (shown in purple). B) Stick type representation: His preserved in position 94, 96, and 119 (shown in green), Thr199 and Tre200 (shown in light blue), binding SUA (4-sulfamoyl-phenyl)-thiocarbamic acid O-(2-thiophen-3-yl-Ethyl) Ester) shown in yellow, and the Zn+2 atom in sphere format. Image adapted from Hassan et al. (12).

III. CARBONIC ANHYDRASE AND CANCER

CA-α isoforms are related to different diseases such as glaucoma (21), epileptic seizures (14), obesity (14), altitude sickness (15), and cancer (15), which makes it a powerful therapeutic target.

Cancer is a heterogeneous group of diseases produced by genetic and epigenetic changes in the cells of human tissue (16). It is one of the health problems that most impacts the general population, and in the United States are the second leading cause of death after cardiovascular disease (17). In Latin America, according to the website of the National Institute of Neoplastic Diseases in Peru, cancer is the third cause of death, and the Registry of Cancer in Metropolitan Lima showed that the incidence rates for all cancers in men and women have increased between the periods between 1968-1970 and 2004-2005 from 152.2 to 174.0 per 100,000 men and from 166.8 to 187.0 per 100,000 women (https://portal.inen.sld.pe/indicadores-anuales-de-gestion-produccion-hospitalaria/). This high mortality rate is attributable to a failure to treat metastatic disease, drug resistance, and in some cases, late diagnosis of the disease. Up to 200 different types of cancer have been recognized (18).

In cancer, tumor cells are divided, generating a hypoxic environment; in other words, low levels of oxygen contribute to local and systematic proliferation (19). Hypoxia induces extracellular acidosis by the anaerobic glycolysis that the tumor cells activate for the use of glycolytic metabolites, producing lactic acid and reducing the pH in the surrounding environment (Warburg effect) (20), (21). These changes in the extracellular pH generate an imbalance that is exploited by the adaptability of the tumor cells that maintain their pH at physiological levels despite being in an acidic environment. It is the CA enzyme, which is in charge of regulating the differential of the pH of solid tumors, allowing the survival of the tumor cells. The non-neoplastic cells cannot adapt to survive in acidic conditions, so they die.

In cancer, it is the isoenzymes CA IX and CA XII that have been extensively investigated because they play an important role in tumor proliferation, acidification, and progression (4,22). Moreover, they have a pH regulation function due to their hydratase activity, regulating the physiological buffer bicarbonate. The over-expression of these isoenzymes has been correlated with different types of cancer. For example, in breast cancer, the presence of high concentrations of mRNAs of CA IX and CA XII has been correlated with the low probability of survival of the patient suffering from it (25).

The CA IX is form encoded by the CA IX gene, detected in HeLa cells and originally called MN or G250(23) protein, is a 414 amino acid long transmembrane glycoprotein with a weight of 58/54 kDa due to post-translational modifications, which forms trimers on its cell surface (24). This domain participates in cell adhesion and is responsible for maintaining catalytic activity in the acidic environment of tumor cells. CA IX is also found in soluble form (s-CA IX) and is present in plasma and urine samples from patients with renal cell carcinoma (RCC) and in plasma from patients with non-small cell lung cancer (NSCLC). This makes s-CA IX a useful marker for the detection of RCC and NSCLC (25). The involvement of CA IX in pH regulation has multiple consequences that support the tumor phenotype and has been reported in different cancers such as cervix, kidney, esophagus, lung, colon, brain, pancreas, liver, breast, endometrium, esophagus, and skin (24, 25).

CA XII isoform was first discovered in RCC by serological expression screening with autologous antibodies. Like CA IX, it is a homodimeric transmembrane glycoprotein formed by 354 amino acids of length and an approximate weight of 44 kDa, which is induced by hypoxia (26). This is expressed in different tissues in a natural way, for example, in the basolateral plasma membrane of epithelial cells of the kidney and pancreas, in epithelial cells of the efferent ducts in men, and in the endometrium in women. CA XII has been reported to be overexpressed in different types of cancer, such as RCC, colorectal, bladder glioblastomas, and head and neck cancers (2, 17, and 27). In the case of breast cancer, the presence of AC XII is indicative of a low degree of disease and a higher probability of recovery in the patient (16).

TABLE I
DISTRIBUTION OF CA IX ISOENZYME IN TUMOR TISSUES

Biodistribution	Testing Method
Tumor Renal cell carcinoma, colon, lung, breast, ovary, head, and neck, pancreatic cancer, transitional cell carcinoma of the urinary tract.	Imaging/detection through AC IX antibodies and AC IX inhibitors immunostaining and WB
Blood renal cell carcinoma non-small cell lung cancer	 ELISA ELISA
Urine Transitional cell carcinoma of the urinary tract	WB

For all of the above, both CA IX and CA XII are over-expressed in hypoxic conditions, in different types of tissues (Table I and Table II) (28), which makes these tissues potential therapeutic targets since their expression is related to a low probability of patient survival (29).

TABLE III
DISTRIBUTION OF CA XII ISOENZYME IN TUMOR TISSUES

Biodistribution	Testing Method
Tumor • kidney • Breast cancer • Lung cancer • Cervical cancer • Ovarian cancer • Colorectal cancer • Brain cancer • Squamous cell carcinoma of the esophagus	 Northem trial Immunostaining Immunostaining Tissue Microarrays immunostaining immunostaining, WB, RT-PCR immunostaining
Blood (mutated version) • Cystic fibrosis-like diseases • Pancreatitis • Sjogren's syndrome	 genotyping, genetic sequencing, RT-PCR immunostaining

Vertebrae • Chronic back pain	Microarray analysis cDNA, RT-PCR immunostaining, flow cytometry
Eyes • Glaucoma	immunostaining, Northem trial

IV. CARBONIC ANHYDRASE AND BIOINFORMATICS

Different techniques have been employed to address the study of Carbonic Anhydrase; one of them, for example, is bioinformatics that has allowed us to understand the evolution and diversity of this enzyme in different species (30-32). It has also allowed us to understand the structural characteristics of carbonic anhydrase inhibitors for the treatment of different diseases (30-34).

Since experimental drug development requires time and funding, the use of computer resources such as the computer-aided design of drugs (CADD) becomes an effective plan for drug development (38). In the constant search for inhibitors for potential targets in cancer treatment, the CA IX and CA XII isoforms are widely studied, by molecular modeling, molecular coupling, pharmacophoric modeling, virtual screening, and machine learning, among others (39). This is due to the accessible and extensive biological information in specialized databases (40, 41).

The development of compounds from natural products is classic in modern medicine, and interest in designing improved inhibitors for CA IX has been proposed through ligand screening. In this context, Coumarin (a natural product that blocks tumor growth) and its derivatives have exhibited a wide pharmacological activity (antimicrobial, analgesic/anti-inflammatory, and anticarcinogenic). Therefore, studies such as the one developed by the group of Dr. Bupesh (35) show the election of the ligand derived from coumarin N-(3,4,5-trimethoxy-phenyl-carbamoyl methyl) of 17 ligands studied as a strong candidate for the inhibition of CA IX due to its high binding energy (~61,58 Kcal/mol) and optimal interaction with catalytic residues (THR 199, PRO 201, HIS 119, HIS 94). Some other compounds derived from coumarin have also been proposed using molecular modeling approaches, for example, 2-oxo-N-((2-(pyrrolidin-1-yl)ethyl)carbamothioyl)-2H-chromene-3-carboxamide, which proved to be the most active compound of 20 possible ligands, interacting with the catalytic amino acids His64, His94, His96 and Gln92 (36).

CA XII inhibitors have also been studied through molecular coupling studies; the selected ligands had a sulfonamide group in common, and to determine the potential of these ligands, the docking score was used to compare them with the one obtained between acetazolamide (comparison pattern) and CA XII. Two ligands had similar standard coupling scores and potential selective affinity for CA XII, the thiophene derivative 4-chloro-N'-(thiophene-2-carbonyl)benzenesulfonohydrazide, and the pyridine derivative 4-methoxy-N'-picolinoylbenzenesulfonohydrazide (42).

Along these lines, Elmira Nazarshodeh and Sajjad Gharaghani, found new possible compounds that could be used for the design of CA XII inhibitors from the proposal of virtual screening using a pharmacophoric model and molecular coupling as well as considering the properties of the drug: Lipinsky's RO5 and the risk of toxicity. The result was 12 non-toxic molecules obtained from the ZINC database (http://zinc.docking.org/) and CoCoCo-MC (http://cococo.isof.cnr.it/), which will provide useful information for further research into the precise design of CA XII inhibitors (37). Inhibitors derived from flavonoid and phenolytic groups for CA II isoforms have also been studied with bioinformatic approaches such as molecular coupling and simulations of molecular dynamics, concluding that the molecules called Physetine found in Dipterocarpaceae and Afr3 found in A. fretessi, present the best coupling score with CA II,~51,46 and ~51.31 kCal/mol respectively (43).

Finally, it is important to emphasize that the possible compounds mentioned above are molecules that can be used as structural bases to design new inhibitors for the AC isoforms described.

V. CONCLUSIONS AND NEXT STEPS: CARBONIC ANHIDRASA MONITORING METHODS

In the last few years, carbon anhydrase has been used as a biomarker for different diseases. Several works have been reported where it is used for lung cancer detection, as mentioned by Lin et al. (48), in which they report a direct relationship between the over-expression of carbon dioxide with lung cancer, assessed the levels of carbon dioxide in serum of 78 people (47 healthy and 31 patients) through the ELISA technique, contrasted with the histological results of each of the patients. In this study, it was demonstrated that there was a higher serum level of carbon anhydrase among patients with lung tumors; in addition, the method presented a sensitivity and specificity of 95.7% and 90.3%, respectively.

ELISA method reveals an excellent monitoring system of carbon dioxide but still presents certain limitations with respect to direct clinical use as high cost, difficult handling, and need for trained personnel. Therefore, other monitoring systems are being developed, such as the one reported by Singh (50), which uses an electrochemical biosensor that allows the recognition of carbon dioxide in a range of 1.08-1.32mM with an acceptable sensitivity with a response time of 1s, proving a promising technology for the application of lung cancer detection.

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