Journal of
Oral Pathology & Medicine

doi: 10.1111/j.1600-0714.2012.01144.x

J Oral Pathol Med (2013) 42: 1–8

wileyonlinelibrary.com/journal/jop

REVIEW ARTICLE

The role of carbonic anhydrase IX in hypoxia control in OSCC

Mario Pérez-Sayáns[1], Claudiu T. Supuran[2], Silvia Pastorekova[3], José Manuel Suárez-Peñaranda[4], Gayoso-Diz Pilar[5,6], Francisco Barros-Angueira[7], José Manuel Gándara-Rey[8], Abel García-García[9]

[1]Oral Medicine, Oral Surgery and Implantology Unit, Faculty of Medicine and Dentistry, Instituto de Investigación Sanitaria de Santiago (IDIS), Santiago de Compostela, Spain; [2]Laboratorio di Chimica Bioinorganica, Università degli Studi di Firenze, Sesto Fiorentino (Firenze), Italy; [3]Institute of Virology, Slovak Academy of Sciences; [4]Servicio de Anatomia Patológica, Hospital Clinico Universitario de Santiago, Choupana s/n Santiago de Compostela, Spain; [5]Clinical Epidemiology and Biostatistics Unit, Hospital Clínico Universitario de Santiago de Compostela, Spain; [6]Instituto de Investigación Sanitaria de Santiago (IDIS), Santiago de Compostela; [7]Unidad de Medicina Molecular – Fundación Pública Galega de Medicina Xenómica, Edificio de Consultas planta, Hospital Clinico Universitario C.P. Santiago de Compostela, Spain; [8]Oral Medicine, Oral Surgery and Implantology Unit, Faculty of Medicine and Dentistry, Entrerríos s/n, Santiago de Compostela, Spain; [9]Oral Medicine, Oral Surgery and Implantology Unit, Faculty of Medicine and Dentistry, Instituto de Investigación Sanitaria de Santiago (IDIS), Santiago de Compostela, Spain

Tumoral microenvironments play a key role in the evolution of solid tumors. Tumor hypoxia is actively involved in the promotion of genetic instability, the invasive capacity of tumor cells, metastasis, and a worsening of the clinical evolution. Endogenous hypoxia markers are controlled by hypoxia-related genes, formed by HIF-1, which is related to several target genes that involve the energy metabolism, angiogenesis, and transmembrane carbonic anhydrases (CAs), mainly CA-IX that is one of the tumor-related carbonic anhydrases. The goal of this paper is to establish the role of CA-IX as a hypoxia marker in OSCC, while analyzing its expression in this type of tumors and its relationship with several clinical and pathological parameters and prognosis, evaluating its relationship with angiogenesis, other hypoxia markers, and clarifying its role in chemotherapy and radiotherapy resistance.
J Oral Pathol Med (2013) **42**: 1–8

Keywords: CA-IX; Carbonic anhydrases; Hypoxia; Oral squamous cell carcinoma

Introduction

Tumoral microenvironments play a key role in the evolution of solid tumors. Hypoxia and tumor cell proliferation determine response to surgery, chemotherapy, and radiotherapy (1–5). Tumor hypoxia is actively involved in the promotion of genetic instability, the invasive capacity of tumor cells, metastasis, and a worsening of the clinical evolution (6–8), resulting in the loss of the apoptotic capacity of cells (9) because of to abnormal tumor vascularization, altered blood perfusion, anomalous oxygen consumption, and anemia (10–13). However, tumor cell proliferation is affected by differentiation status, cell-cycle regulation, and microenvironmental factors – including the availability of oxygen and nutrients (14, 15). Hypoxia delays tumor cell proliferation maintaining cell superpopulations capable of proliferating under hypoxic conditions, responsible for treatment failure (16), as has been confirmed by authors such as Hoogsteen et al. (17) in head and neck carcinomas (HNSCC).

Among the hypoxia markers, we will focus on the exogenous hypoxia markers, mainly 2-nitroimidazole, pimonidazole, and EF-5, which are accumulated upon administration in tumor hypoxic areas and can be visualized after tumor removal (18, 19). In addition to exogenous markers, there are endogenous hypoxia markers, controlled by hypoxia-related genes, formed by HIF-1 (20, 21), which is related to the von Hippel-Lindau (vHL) tumor suppressor protein during oncogenesis (22) and which also controls several target genes that involve the energy metabolism (glucose and glycolytic enzyme transporters) (23), angiogenesis (VEGF) (24) and transmembrane carbonic anhydrases (CAs), mainly CA-IX (25, 26). The hydroxylation of HIFα and its regulation by the von Hippel-Lindau protein (VHL) under normoxia or hypoxia are responsible for regulating the activation or inactivation of these HIF-dependent genes, involved in different aspects related to carcinogenesis (Fig. 1).

CAs are transmembrane Zn metalo-enzymes that catalyze reversible hydration of carbon dioxide in

Correspondence: Mario Pérez-Sayáns, Oral Medicine, Oral Surgery and Implantology Unit, Faculty of Medicine and Dentistry, Instituto de Investigación Sanitaria de Santiago (IDIS), Entrerríos s/n, Santiago de Compostela, C.P. 15782 Spain. Tel: +0034626233504, Fax: +0034986295424, E-mail: perezsayans@gmail.com
Accepted for publication February 16, 2012

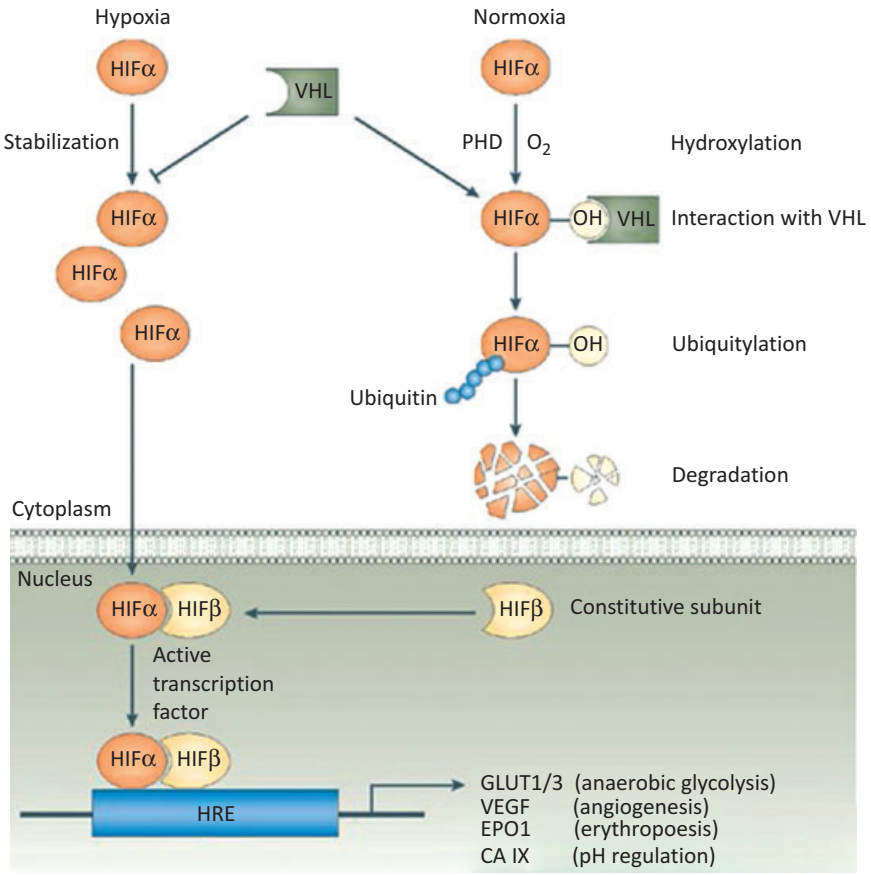

Figure 1 Mechanism of hypoxia-induced gene expression mediated by the HIF transcription factor. At normal oxygen levels (normoxia), prolyl-4-hydroxylase (PHD) hydroxylates the P564 on hypoxia inducible factor-α (HIFα). The von Hippel-Lindau protein (VHL) binds hydroxylated HIFα and targets it for degradation by the ubiquitin–proteasome system. Under hypoxia, HIFα is not hydroxylated, because PHD is inactive in the absence of dioxygen. Non-hydroxylated HIFα is not recognized by the VHL protein; it is stabilized and accumulates. After translocation to the nucleus, HIFα dimerizes with the HIFβ constitutive subunit to form an active transcription factor. The HIF transcription factor then binds the hypoxia response element (HRE) in target genes and activates their transcription. Target genes include glucose transporters (GLUT1 and GLUT3) that participate in glucose metabolism, vascular endothelial growth factor (VEGF) that triggers neoangiogenesis, erythropoietin (EPO1) involved in erythropoiesis, carbonic anhydrase (CA) IX involved in pH regulation and tumorigenesis, and additional genes with functions in cell survival, proliferation, metabolism, and other processes (25).

carbonic acid and are involved in respiration and acid-base equilibrium (27). There are 14 known members of this family, which are subdivided according to their location: membrane-related, cytosolic, mitochondrial, and secreted (28). CA-IX and CA-XII are the two tumor-related carbonic anhydrases (29, 30), although CA-IX is largely expressed in tumor cell lines and shows moderate-low expression in a healthy gastrointestinal tract, hence its high specificity as a tumor hypoxia marker (31, 32). CA-IX expression has been thoroughly described in different tumors, including cervical carcinoma (33), lung (34), bladder (35), breast (36), esophagus (37), and colorectal cancers (38); however, this has not been so for head and neck carcinomas and, more specifically, in the case of oral squamous cell carcinomas (OSCC), which account for 95% of oral malignant neoplasms (39).

The goal of this paper is to establish the role of CA-IX as a hypoxia marker in OSCC, while analyzing its expression in this type of tumors and its relationship with several clinical and pathological parameters and prognosis, evaluating its relationship with other hypoxia markers, and clarifying its role in chemotherapy and radiotherapy resistance.

CA-IX expression and prognosis in OSCC

As we have established above, CA-IX expression is located in the plasma membrane, solely and exclusively in tumor cells; in some cases, tincture forms a continuous reticule that surrounds the contour of the cell, in such cases, expression is strong; however, it tends to be diffuse in neoplasms, mainly in the center of tumor nests. The second pattern is similar, but membrane tincture is incomplete, weak, and limited to the periphery of the tumor (40–44). The expression results for the different studies are summarized in Table 1.

As for the relationship with clinical and pathological parameters, and especially with prognostic factors, the results are variable depending on the series of studied cases, as is the case of other markers of this type of tumors (5, 42). According to Choi et al. (40), CA-IX

Table 1 CA-IX Immunohistochemical expression in OSCC

Study	*OSCC Cases*	*%* (n) *Positivity*				*%* (n) *Negativity*	*Quantification*
(40)	117	58.1% (68) 54 (1+)		14 (2+)		41.9% (49)	CA-IX (0) <5% CA-IX (1+) 5–20% CA-IX (2+) >20%
(44)	107	98% (105)				2% (2)	CA-IX (−) <10% CA-IX (+) ≥10%
(45)	80	42.5% (34) 21 low		11 mod	2 intense	57.5% (46)	CA-IX (1) 1–10% CA-IX (2) 11–50% CA-IX (3) 51–80% CA-IX (4) >80%
(41)	43	40.47% (26) 7 (1+)	7 (2+)	10 (3+)	2 (4+)	39.53% (17)	CA-IX (0) 0% CA-IX (1+) 1–10 CA-IX (2+) 11–50% CA-IX (3+) 51–80% CA-IX (4+) 81–100%
(46)	60	CA-IX <10%: 36.7% (22) CA-IX ≥10%: 63.3% (38)					CA-IX <10% CA-IX ≥10%

ND, not determined; mod, moderate.

expression is related to post-surgical recurrence and a worse average survival rate, and therefore consider it as a good prognostic marker. For Kondo et al. (44), CA-IX expression is positive in 98% of tumors, finding lower survival in patients with elevated CA-IX expression (≥50% of cells). Furthermore, patients with poorly differentiated tumors, T4, lymph-node metastasis, and stage IV with high CA-IX expression showed a worse outcome. For Kim et al. (46), the percentage of positive cells ranges between 0 and 77.5 percent. In their series of OSCCs of the tongue, they found a relationship between high CA-IX (≥10%) expression and poorly differentiated tumors, with those located in the base of the tongue of smokers and patients who had been submitted to radiotherapy in contrast with those who had only undergone surgery.

In contrast, Roh et al. (41) found that CA-IX levels were moderate to high in a sample of 43 OSCC patients, establishing a positive correlation only with tumor thickness, without affecting their overall survival nor the 5-year disease-free period. Eckert et al. (45) only found a greater expression of CA-IX in women, without relating such expression to prognostic factors.

CA-IX expression in other HNSCCs.

Although hypoxia phenomena participate in the same way in all HNSCCs, the results of CA-IX expression differ from those of OSCC studies; some authors have even described CA-IX cytoplasmic expression (47, 48). Hoogsteen et al. (17) analyzed CA-IX expression and a cell proliferation marker (iododeoxyuridine: IdUrd) in a series of 60 HNSCC cases, including 3 OSCC cases. In this study, they found that joint expression of CA-IX (variable 0–39%) and IdUrd (0–81%) is related to cell subpopulations responsible for repopulation and disease progression. These cells were found especially at an intermediate distance from blood vessels (100–150 μm) and showed a relation between these tumors and a shorter disease-free period. Le et al. (48), in a study with 101 HNSCC cases, found an elevated correlation of hypoxia levels and CA-IX; however, the latter was not related to any of the prognostic variables. Along the same line, Kaanders et al. (49) studied the distribution of pimonidazole (exogenous hypoxia marker) and CA-IX in 43 HNSCC cases, observing expression fundamentally at a short distance from blood vessels and with a positive correlation. Furthermore, patients with hypoxic tumors and low vascular density showed worse locoregional control, although no relation was found between CA-IX expression and treatment outcome. However, such associations disappear when patients are treated with ARCON (accelerated radiotherapy combined with carbogen and nicotinamide).

HNSCCs, which are hypoxic tumors by definition, are frequently diagnosed in very advanced stages, especially in HPV-positive cases that normally have better prognosis (50). Certain authors, like Brockton et al. (51), hypothesized on the control of endogenous hypoxia markers by oxygen-independent factors; therefore, they studied their relation with CA-IX expression, and not HPV and p16. Their results showed that a high stromal expression of CA-IX is related to a reduced average survival in p16-negative tumors. According to Kong et al. (52), 44% (36 of 82) HNSCC cases under study presented a strong HPV pyrosequencing signal; however, they found no relation with tumor hypoxia and CA-IX expression.

Relationship between CA-IX and chemoresistance or radioresistance

The relationship between CA and blood vessels has been described by several authors; thus, Koukourakis et al. (47), in series of 75 HNSCC cases that were treated with chemotherapy and radiotherapy, observed that CA-IX expression (26.6%, 20 of 75) takes place mainly in tumors with low vascularization (measured by microvascular density (MVD), positive for CD-31), necrosis areas, and is related to a poor overall response. These results were confirmed by Jonathan et al. (53) and Beasley et al. (54); the latter in three

HNSCC cell lines and 79 specimens (31 OSCCs). The average CA-IX expression was 20% (0–90%) and was induced by cell line hypoxia and was related to necrotic areas, high MVD (positive for CD-34), and advanced tumor staging; the average distance between blood vessels and the bottom line of the expression was 80 μm (40–140), thus confirming the results of Hoogsteen et al. (17). A study of a HNSCC xenograft, conducted by Bhattacharya et al. (55), confirmed the lack of microvessels in well-differentiated areas of the xenograft related to hypoxia and positive for CA-IX (detected by functional MRI), a limited use of chemotherapeutic drugs, and resistance to Irinotecan therapy, thus confirming the hypothesis that hypoxia promoted the creation of resistant cell subpopulations. This same team tried to improve their results by adding tirapazamine (a chemotherapeutic drug with selective toxicity for hypoxic cells), but the results were not what they had expected, as it resulted in a reduction of blood vessels, thus reducing drug dosage in CA-IX-positive cells in hypoxic regions (56). These results were confirmed by Chintala et al. (57), who studied the effect of Se-methylselenocysteine (molecule that increased the effect of irinotecan) in HNSCC cell lines and xenografts. They observed that, in cells and hypoxic areas, the combination of both drugs reduced HIF-1α levels, which, at the same time, transcriptionally regulated and lowered CA-IX levels. The hypothesis that CA-IX actively participates in chemoresistance has been confirmed by Zheng et al. (58). In their research, they transformed an OSCC of the tongue cell line, which was moderately differentiated, into pingyangmycin resistant (PYM) (Tca8113/PYM) and cross-resistant to paclitaxel, Adriamycin, and mitomycin. It was confirmed that neither glycoprotein p (p-gp), or multidrug resistance-associated protein 1, or breast cancer resistance protein were involved in the acquired resistance. To verify the responsible factors, they analyzed cell lines by DNA microarray, PCA, and Western Blot, and found that application of CA inhibitor, acetazolamide, and CA-IX silencing with oligonucleotides contributed to increase average pH in resistant cells, thus resulting in an increase of chemosensitivity to PYM, in addition to increasing activation of PYM-induced caspase 3. Currently, the possibility of using tumor-associated antigens (TAA) such as G250/CA-IX for immunotherapy in HNSCC with up to 80% protein expression levels to produce a specific response of T $CD8^+$ cells is under study (59).

As regards to the role of CA-IX in radiotherapeutic treatments, Eriksen et al. (60) tried to determine its role as a prognostic marker in a series of 320 HNSCCs undergoing radiotherapy treatments with concomitant nimorazole, a hypoxia-modifying drug. The research findings established that CA-IX is not related to any clinical and pathological, prognostic (outcome and disease-free period) parameters; it was also proven useless as a marker for concomitant use of radiotherapy + nimorazole. As we have mentioned above, in the case of patients treated with ARCON vs. conventional surgery ± radiotherapy, the hypoxia and vascular density levels have no influence on treatment response (49). These same results were found by Jonathan et al. (53) who reported that the relationship between CA-IX (expression >25% of tumor area) and the lack of locoregional control and freedom from distant metastasis and their relation with GLUT-3 disappears when tumors are treated with ARCON. According to Koukourakis et al. (61), joint expression of HIF-2α and CA-IX is responsible for poor CHART (continuous hyperfractionated accelerated radiotherapy) results, in contrast with conventional radiotherapy.

The relationship between CA-IX and other molecules

HIF

Dimeric HIF-1α transcription is the regulating factor in cellular response to hypoxia (62, 63), activating several genes (over 60) (64) including genes coded by vascular endothelial growth factor (VEGF), erythropoietin (EPO), and several enzymes in the metabolism of glucose, iron, and nucleotides (65). In the case of OSCC, HIF-1α prevents apoptosis of tumor cells (66); however, its relationship with other endogenous markers, such as CA-IX, remains unclear. Zhu et al. (67) found that when OSCC cell lines are cultivated with 1% O_2 expression of mRNA CA-IX is regulated by HIF-1α, rather than HIF-2α. Chintala et al. (57) confirmed the reduction of CA-IX following reduction of HIF-1α by application of Se-methylselenocysteine combined with irinotecan. Koukourakis et al. (61) found a relationship between CA-IX expression and worse survival rates, and between HIF-2α and a worse locoregional control in a series of 198 HNSCCs (33 OSCCs). Both were independent prognostic factors, but their joint expression provoked an additive effect, thus confirming the relation between both markers.

As regards to the clinical and pathological parameters, Eckert et al. (45) studied the relationship between two of the most important hypoxia markers, HIF-1α and CA-IX. Surprisingly, they found that patients with low expression of both proteins survived an average of 54.8 months, while those with high HIF-1α and low CA-IX expression survived an average of 37.6 months and their tumor-related death risk was 4.97-fold. Roh et al. (41) also found a statistically significant relationship between CA-IX and HIF-1α ($P = 0.005$) and HIF-2α ($P = 0.029$) expression. However, Winter et al. (68) found CA-IX expression in 56 of 149 HNSCC cases; however, they did not confirm a positive correlation with HIF-1α nor HIF-2α.

Ki-67

Kim et al. (46) studied the relationship between CA-IX and the Ki-67 proliferation factor. They observed a correlation between both ($r = 0.373$, $P = 0.0008$), thus establishing a risk model based on the expression of both factors. They thus established three patterns: high risk (elevated CA-IX/Ki-67), low risk (low CA-IX/Ki-67), and moderate risk (one of the two is elevated). The high-risk group was an independent prognostic factor for average survival and disease-free survival. On the

other hand, Kondo et al. (44) found no relationship between Ki-67 expression and CA-IX.

GLUT-1

Schutter et al. (69) studied the expression of CA-IX and GLUT-1 (glucose transporter-1) in biopsies of tumors that had been previously treated with radiotherapy ± chemotherapy, in a series of 67 HNSCC cases (2 OSCCs). CA-IX expression accounted for an average of 17.14% (0–87.8%) and its expression was not related to any of the clinical, pathological, or prognostic parameters, nor with the expression of GLUT-1 (mean 67.5%, range: 0–100%). However, in patients showing above average joint expression of CA-IX and GLUT-1, this was an independent prognostic factor for local control and disease-free period. Meanwhile, once again, neither Kondo et al. (44) nor Jonathan et al. (53) found relation between GLUT-1 expression and CA-IX.

Erythropoietin (Epo) and erythropoietin receptor (EpoR)

Certain authors, like Winter et al. (70), have tried to link tumor hypoxia and its main marker, CA-IX, with hemoglobin (Hb), Epo, and EpoR levels. Thus, in a series of 151 HNSCCs (51 OSCCs) they found a positive correlation in the expression of Epo and EpoR, and CA-IX and Epo, but not EpoR. There was no correlation between Hb and Epo or EpoR; therefore, tumoral anemia does not seem to regulate the hypoxic conditions of the microenvironment nor Epo levels.

Other molecules

Le et al. (48) found a significant relationship between intense CA-IX expression and hypoxia-related proteins: BNIP3L (BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like), LOX (lysyl oxidase), CTGF (connective tissue growth factor), Ephrin A1, and GAL-1 (Galanin receptor-1). Similarly, Le et al. (71) confirmed in a series of 101 HNSCCs, cell lines, and xenografts, a positive correlation between the expression of CA-IX and Galectin-1 (a hypoxia-induced secreted protein) and their relation with treatment outcome. Silva et al. (72), in a series of 60 HNSCC patients (33 in tongue), found that co-expression of CA-IX and MVP (major vault protein) confers tumors a significantly lesser chance of locoregional control. According to Gee et al. (73), there are abnormally high levels of microRNA hsa-miR-210 in 46 HNSCC patients (10 OSCC cases) with a statistically significant relationship between hypoxia markers such as HIF-1α, the TWIST1 gene, and carbonic anhydrase IX (CA-IX), with locoregional recurrence with a smaller average survival. Schutter et al. (74), analyzing the relation between micro-satellite alterations and HNSCC tumor hypoxia, found that LOH (loss of heterozygosity) is very frequent in regions close to p53, specifically in D17S799, in patients that simultaneously showed high CA-IX expression ($P = 0.01$), thus supporting the theory of the relationship between p53 and hypoxia (9). However, Kondo et al. (44) found no relationship between p53 expression and CA-IX.

Role of CA-IX as therapeutic target against cancer

Isozymes CA-IX and XII are predominantly found in tumor cells and show a very restricted expression in normal tissues (26, 27). It has been recently proven that by efficiently hydrating carbon dioxide to protons and bicarbonate, these CAs contribute significantly to the extracellular acidification of solid tumors (in addition to lactic acid), whereas their inhibition reverts to a certain extent this phenomenon (26). CA-IX is overexpressed in many tumors in response to the HIF pathway (21), and research on the involvement of these isozymes in cancer has progressed significantly in recent years, allowing design campaigns of inhibitors against this novel, recently validated antitumor target (75). Several approaches were discovered in the last years for obtaining compounds that specifically target the tumor-associated isoforms CA-IX and XII (which are extracellular proteins, with their active site outside the cell), among which, coumarin and thiocoumarins are the most important such new CAIs.

But probably the most interesting CAIs reported to date are the ureido-sulfonamide and the glycosyl coumarin (76, 77). Both of them are low nanomolar CA-IX-selective inhibitors, which strongly inhibit the growth of both primary tumors and metastases in several animal models of breast cancer. In these straightforward studies, a similar animal model of breast cancer cell lines which does not express CA-IX has been used as negative control (cell line 67NR), and no effects on the growth of the tumors have been evidenced after treatment with sulfonamide/coumarin CAIs and (76, 77). These data undoubtedly demonstrated the potential of CA-IX inhibition for designing antitumor/antimetastatic agents possessing a novel mechanism of action.

Apart small molecule inhibitors, M75 is a highly specific anti-CA-IX mAb targeting the PG domain of CA-IX, (78, 79). It has been highly used in immunohistochemical and western blot studies for evidencing CA-IX in various types of tumors, but also radiolabelled with ^{125}I to use it as a tool for tumor imaging by means of positron emission tomography (PET) (79). WX-G250 (Girentuximab) is another anti-CA-IX chimeric monoclonal antibody in phase III clinical trials as adjuvant therapy for the treatment (by once-weekly infusion) of non-metastasized renal cell carcinoma (RCC) in patients at high risk of recurrence after resection of the primary tumor (80).

Although these molecules have not been used in OSCC, all these data demonstrate that the tumor-associated CAs are indeed almost ideal targets for designing novel and innovative anticancer drugs which interfere with tumor acidification by a mechanism of action not yet exploited by the classical cytostatic drugs.

Conclusions

It is clear that hypoxia in solid tumors is a decisive factor for the outcome of HNSCCs, and especially OSCCs. However, despite the fact that the regulating

endogenous markers have been perfectly described, the relevance of each one of them, especially CA-IX and their inter-relations, has not been strongly confirmed. Probably this is due to the expression results in each of the different studies and their relationship with clinical and pathological parameters, as well as prognostic factors, which present great variability, resulting in a reduction of scientific evidences. These evidences, however, exist when relating hypoxia in solid tumors with chemoresistance and the failure of radiotherapy, both conventional and concomitant, in which CA-IX seems to play an important role. Several mAbs (girentuximab, and its ^{124}I-radiolabelled variant) targeting CA-IX are in advanced clinical trials both for the treatment and for imaging of hypoxic tumors overexpressing this enzyme. These trials seem to be highly successful and presumably soon these mAbs will be approved for clinical use. Two small molecule CA-IX inhibitors, the sulfonamide and the glycosyl coumarin, are also in advanced preclinical evaluation at this moment, both for imaging and treatment of solid tumors and metastases in which CA-IX is present. We consider that further studies of these tumors are needed to confirm the use of CA-IX as a prognostic marker and to evaluate its possible inhibition with minimal adverse effects, reducing the risk of metastasis, and favoring the action of chemotherapeutic drugs and radiotherapy in OSCC.

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