Role of V-ATPases in solid tumors: Importance of the subunit C (Review)

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Abstract. Acidity is one of the main characteristics of OSCC (oral squamous cell carcinoma) as a solid tumor. The V-ATPase is the primary regulator of the tumor micro-environment, by means of proton extrusion to the extracellular medium. The decrease in extracellular pH confers the cells a resistant, highly invasive and metastatic phenotype. However, the acid medium confers an optimum pH to the degradative enzymes (such as proteases and MMPs) for their proper functioning. The C subunit (ATP6V1C) of V1 intra-membrane domain of the V-ATPase, is primarily responsible for its enzymatic function, through the control of a reversible dissociation of V0 and V1 domains. In this review, we describe the importance of V-ATPases in the control of tumor micro-environment, the potential strategies as protein targeting to improve the effectiveness of drug treatment and the role of the C subunit as the primarily responsible of the enzymatic control. The inhibition of the V-ATPase activity through PPIs (proton inhibitors) seems to reduce the destructive and metastatic capacity in tumors, such as hepatocellular carcinoma. Nevertheless, none of these inhibitors was proven to be useful in OSCC; therefore, it is highly important to carry out further studies in order to develop specific inhibitors of the C subunit, to control the devastating effects of OSCC.

Contents

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1. Introduction

The main characteristics of the solid tumors (such as oral cancer) are the acidity and hypoxia, phenomena that result from the progression of metastatic cancer (1), the sensitivity to chemotherapeutic agents (2) and proliferation (3). In fact, a mechanism of resistance to cytotoxic drugs is the alteration of the pH gradient between the extracellular environment and cell cytoplasm (4).

The cytosolic pH seems to be strictly regulated by four mechanisms: the family of sodium-proton exchangers (NHE), the family of bicarbonate transporters (BCT), the family of monocarboxylate transporters (MCT) and the proton pumps (ATPase) (5,6) (Fig. 1). The lactate production has been commonly seen as the first acidification mechanism of the microenvironment (7). The lactate accumulation results in the activation of the aerobic glycolytic metabolism (8) which increases the amount of cellular lactate that is transported outside the cell through the H^+/lactate co-transporter (MCT) (9). The increase in aerobic glycolysis (8,10) provides to the tumor a metabolic environment characterized by low levels of serum, hypoxia and an acid extracellular pH. This micro-environment increases the invasive ability of the tumor and the expression of growth and angiogenic factors/receivers (11). All this is correlated to an increment of the intracellular pH, an aggravation of the initial development of the interstitial acid microenvironment and a reversed transmembrane pH gradient (11,12). This increase in the intracellular pH is concomitant with an increment of DNA synthesis (8,13,14), cell cycle progression (15-17), serum and substrate-independent growth (8) and the *in vivo* growth of the tumor (8,18) and all these phenomena trigger a pathological and disorganized increase in density and cell number. However, tumors are able to create an acidic environment even in conditions of reduced production of lactate, suggesting that the aerobic metabolism is not the major mechanism responsible for the development of an acidic microenvironment within solid tumors such as oral squamous cell carcinoma (OSCC) (19,20). On the one hand, the same favourable conditions are maintained for the tumor cells, and on the other hand the selection of highly malignant cancer cells (which can survive in a hostile environment) is facilitated (21).

To survive in this microenvironment, tumor cells must have a regulatory system of cytosolic pH that assists cells in defending themselves against the dangerous H^+ ions. This

could explain the fact that the V-ATPases, which normally reside in acidic organelles may be also located at the cell surface, regulating the pH and exacerbating the migratory ability of metastatic cells (22,23).

2. Role of V-ATPases in oral cancer

The cell transformation and carcinogenesis processes are accompanied by metabolic disorders, increased production of protons, acidification of the extracellular environment and alkalization of the cytoplasm (24-26). Therefore, the development and maintenance of this gradient is due directly to the ability of tumor cells to secrete protons (H^+), acidify the extracellular environment (11,12,26) and maintain an alkaline cytosolic pH (27). In addition, this ability is increased with the aggressiveness of the tumor (28,29).

Immunohistochemical data show that the V-ATPase is located in the plasma membrane of breast (23,24) and lung (30) cancer cells; this occurs prominently in the highly metastatic cells and with less intensity in the lowly metastatic cells (23,24). Otero-Rey *et al* demonstrate the ATP6V1C1 overexpression in OSCC, one of the most significant subunit of the V-ATPases (31).

According to Martínez-Zaguilán *et al* (24) the V-ATPase expression in the plasma membrane is due to some kind of dysfunction of the normal constituents of the cell. These constituents include the cytoskeleton (32), leader sequences (33) or alterations in a chaperone (34). To Sennoune *et al* the changes in cytosolic pH have multiple phenotypic expression, but according to the authors, the alteration of a single protein (the V-ATPase in the plasma membrane) is responsible for the dysfunctions in protein and cellular pathways of cancer (27).

The acid component of the intratumoral metabolic microenvironment increases the metastatic potential by promoting the angiogenesis (27,35), the anchorage-independent growth, the genetic instability (7) and the invasion, infiltration and penetration of cancer cells into the normal tissue (11).

Martínez-Zaguilán *et al* found that microvascular endothelial cells with the highest migratory capacity express V-ATPases in the plasma membrane. The treatment of these cells with inhibitors of V-ATPases reduces the proton flux, via inhibition of V-ATPase in the plasma membrane (pmV-ATPases) and cell migration, suggesting that they are essential for the regulation of cytosolic pH and migration of endothelial cells (36).

The proton flux, via V-ATPase, evaluated by fluorescence spectroscopy in living cells, was greater in highly than in lowly metastatic cells. Curiously, the lowly metastatic cells use preferably Na^+/H^+ and HCO3- transporters, while highly metastatic cells use V-ATPases. Moreover, these latter cells are more invasive and migratory than the former. These data indicate that the pmV-ATPases are involved in the acquisition of a more metastatic phenotype (37).

The V-ATPases play an important role in the development of tumor metastasis, as previously said. Many tumor cells secrete lysosomal enzymes, involved in the degradation of the extracellular matrix, required for metastatic invasion. These enzymes have a low optimum pH and the V-ATPases are the only responsible for the microenvironment acidification (24,38).

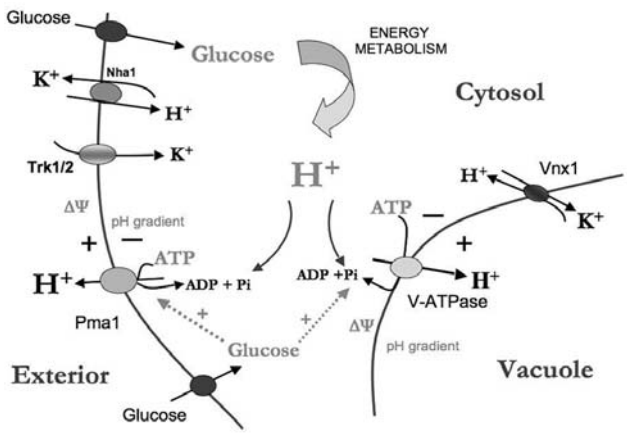

Figure 1. Role of the V-ATPase in the control of intra and extracellular pH. Obtained from Martínez Muñoz and Kane (76).

The motility and invasive phenotype are the requirements that make the cell responsible for metastasis (11,27).

The treatment with V-ATPase inhibitors (such as Bafilomycin A1 and Concanamycin A) inhibits the enzyme activity as well as the migratory ability of highly metastatic cells (23,30). It has been shown that the greatest increase in the invasiveness of tumor cells is the result of two complementary mechanisms: breaking of the cell-matrix interactions that arise because of the acid secretion increment, the protease activity (such as Cathepsin B) and the increased cell motility (11,23,30). The breast cancer cells, through V-ATPases, acidify the extracellular environment in order to facilitate the reabsorption of the extracellular matrix by means of proteases and metastasis (28).

Cell pH is crucial for several biological functions such as cell proliferation, invasion and metastasis, drug resistance and apoptosis. The hypoxic conditions are frequent phenomena during the OSCC development and they cause an intra- and extracellular acidosis. This cellular acidosis seems to be a trigger for apoptosis and allows the endonuclease activation that induces DNA fragmentation. The pH regulators should be over-regulated in the tumor cells in order to avoid intracellular acidification under the above-mentioned conditions (31).

As already mentioned, the tumor microenvironment is essential for the neoplastic progression and the reduction of extracellular pH is one of this microenvironment features. Since V-ATPase is the main proton pump regulator of the cell pH, its involvement in the neoplastic progression should not surprise anyone.

3. V-ATPases as protein targeting

Growing scientific evidence suggests a key role of tumor acidic microenvironment in cancer development, in terms of progression and metastasis. Among all regulatory mechanisms of tumor microenvironment, the V-ATPases play a key role due to their inhibition possibility by means of RNA interference techniques and inhibitors of proton pump (39).

Early attempts to block the V-ATPases as a protein targeting date back to 1988, when Moriyama *et al* described the inhibition of V-ATPase activity, through blocking the

Table I. Inhibitors of pH regulators.

A. V-ATPase inhibitors
Bafilomycin A1
Concanamycin A (Folimycin)/B
NEM: N-ethyl-maleimide
NBD-Cl: 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole
DCCD: N,N0-dicyclohexyl-carbodiimide
Destruxin B
Salicylihalamide A
Lobatamide
Oximidine
B. NHE inhibitors
Guanidine derivatives
(i) Benzoylguanidine
Cariporide, HOE642: 4-isopropyl-3-methylsulphonylbenzoyl-guanidine methanesulphonate
Hoe 694: 3-methylsulfonyl-4-piperidinobenzoyl, guanidine hydrochloride
FR183998: 5-(2,5-dichlorothiophen-3-yl)-3-[(2-dimethylaminoethyl)carbamoyl]benzoylguanidine dihydrochloride
FR168888: 5-hydroxymethyl-3-(pyrrol-1-yl) benzoylguanidine methanesulfonate
EMD 85131: 2-methyl-5-methylsulfonyl-1-(1-pyrrollyl)-benzoylguanidine
(ii) Carbonylguanidine
Zoniporide or CP-597,396: [1-(Quinolin-5-yl)-5-cyclopropyl-1H-pyrazole-4-carbonyl]guanidine hydrochloride monohydrate
TY-12533: 6,7,8,9-tetrahydro-2-methyl-5H-cyclohepta[b]pyridine-3-carbonylguanidine maleate
CAS 181048-29-3, MS-31-050: 2-(2-methylphenyl)-5,7-dimethoxy-4-quinolyl carbonylguanidine dihydrochloride
CAS 181048-36-2, MS-31-038: 2-phenyl-8-(2-methoxyethoxy)-4-quinolyl carbonylguanidine bismethanesulfonate
KB-R9032: N-(4-isopropyl-2,2-dimethyl-3-oxo-3,4-dihydro-2H-benzo[1,4]oxazine-6-car bonyl)guanidine (4b) methanesulfonate salt
(iii) Others
T-162559: (5E,7S)-[7-(5-fluoro-2-methylphenyl)-4-methyl-7,8-dihydro-5(6H)-quinolinylideneamino] guanidine dimethanesulphonate
Amiloride derivatives
DMA: 50-(N,N-dimethyl)-amiloride
HMA: 5-(N,N-hexamethylene) amiloride
MIA: 5-(N-ethyl-N-isopropyl)-amiloride
C. Bicarbonate transporter inhibitor
Triflocin: 4-(a,a,a-trifluoro-m-toluidino)-nicotinic acid
DIDS: 4,40-diisothiocyanato-stilbene-2,20-disulfonic acid
SITS: 4-acetamido-40;isothiocyanostilbene-2,20-disulfonic acid
S3705
D. MCT inhibitors
DIDS: 4,40-diisothiocyanato-stilbene-2,20-disulfonic acid
a-cyano-4-hydroxycinnamate (a-CHC)
p-Chloromercuribenzenesulphonate
Diethyl pyrocarbonate
Quercetin

assembly and reducing H^+ secretory activity, using fusidic acid and suramin (40).

In 2001, Boyd *et al* described a small group of molecules that share a common core of benzyl-lactate-enamide in their structure. These molecules are proton pump inhibitors (PPI) (Table I). The most representative are the following: *salicylamide A*, *lobatamides A-F*, *oxymidines I* and *II*, *bafilomycins* and *canamycins*. Of these, the authors found that the latter two are the most potent V-ATPase inhibitors and even that there is no distinction between mammalian and non-mammalian V-ATPases. This inhibition causes a reduction in the development of tumor cells and cell lines with oncogenes (41) through programmed cell death (apoptosis) (42). The PPI effect is mediated by a very early production of reactive oxygen species (ROS) that preceded alkalinization of lysosomal pH, lysosomal membrane permeabilization, and cytosol acidification, suggesting an early destabilization of the acidic vesicular compartment. Lysosomal alterations were followed by mitochondrial membrane depolarization, release of cytochrome c, chromatin condensation, and caspase

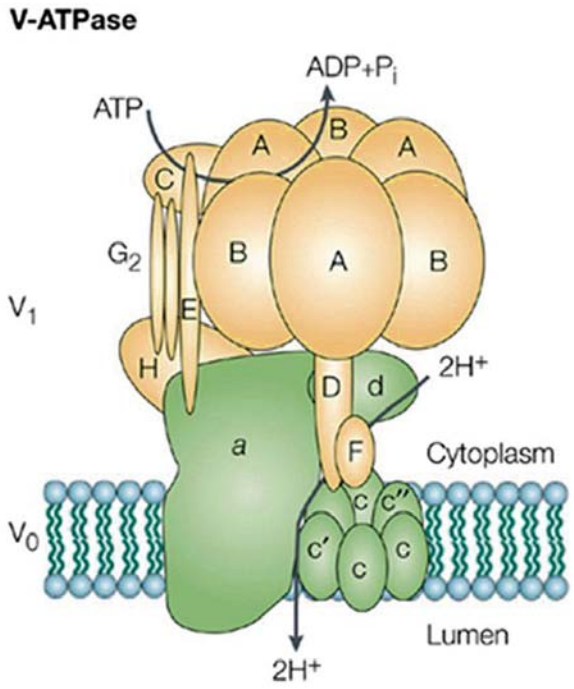

Figure 2. The cytosolic domain V1 (shown in yellow), is composed of three subunits A and three subunits B, two subunits G and one subunit of types C, D, E, F and H. The transmembrane domain Vo (shown in green) is composed of five different subunits: a, c, c', c'' and d. ATP hydrolysis by the peripheral V1 domain drives proton transport through the integral V0 domain from the cytoplasm to the lumen. Modified from Nishi and Forgac (37).

activation (43). It seems that the ROS have a clear effect on oral carcinogenesis (44).

Regarding B-cell lymphomas, the proapoptotic activity of PPI is clearly related to the inhibition of tumor growth (43).

Yoshimoto and Imoto show new roles of V-ATPase inhibitors in tumor cells that overexpress EGFR (epidermal growth factor receptor). The authors find that the V-ATPase inhibitors induce apoptosis in EGFR-stimulated A431 cells (human epidermal carcinoma) with the same dose range that inhibits the V-ATPase activity. However, when cells are not stimulated by EGFR, the inhibitors do not induce apoptosis, while they put a brake on cell growth, independently of the cell cycle (45).

The ATP6L or the C subunit of V0 domain has been determined in recent studies as a possible target in the suppression of metastasis and tumor growth via V-ATPase inhibition, aimed at altering the acid microenvironment of the extracellular matrix, which is necessary for the activity of many MMPs (metalloproteinases) and proteases (23,27,46,47).

Saroussi and Nelson (23) and Ohta *et al* (48) note that V-ATPase C subunit emerges as overexpressed in pancreatic invasive tumors when compared to benign or non-invasive tumors, suggesting that the V-ATPase may have a key role in tumor progression.

However, Otero-Rey *et al* find that ATP6V0C is not overexpressed in OSCC in a statistically significant way, so that blocking this gene does not seem to be very useful in this type of tumors (31).

Lu *et al* demonstrate the ability to slow tumor growth and to suppress distant metastasis in human hepatocellular carcinoma, by the decrease of proton extrusion and the activity of the gelatinase, via inhibition of C subunit gene (ATP6L) by using RNA interference techniques (47).

It has been shown that the E5 oncoprotein of the bovine papillomavirus binds to the C subunit of V-ATPase V0 domain (49,50), triggering a Golgi alkalization that correlates with cell transformation induced by this protein (51); therefore, blocking the V-ATPase via inhibition of C subunit could suppress the carcinogenic effects of HPV.

It seems that Concanamycin A binds to the C subunit of the V0 (52) domain as well as the Bafilomycin (53). According to Bowman (75) the mutations in the genomic sequence of the C subunit (four mutations have been found: T32I, F136L, Y143H and Y143N) give the cell the ability to resist Bafilomycin A1.

There are other new specific inhibitors of the V-ATPases, which are already synthesized in the laboratory as the analogues of archazolid A and B (54). The use of the novel NiK12192 V-ATPase inhibitor increases the anti-tumor activity of other chemotherapeutic drugs (23,55). Another study shows the efficacy of FR202126 (a specific V-ATPase inhibitor of osteoclasts) in decreasing the osteolysis in the lung cancer metastasis (56).

However, it seems that none of these inhibitors has been proven useful in the OSCC, so that it is of high importance to carry out further research in order to determine the actual implication of V-ATPases in cancer development and implementation of other inhibitors in the subunits responsible for enzyme assembly (23).

4. Importance of C subunit

V-ATPase is composed of a cytosolic V1 domain and a transmembrane V0 domain. The V1 domain consists of three A and three B subunits, two G subunits as well as a C, D, E, F and H subunit. C subunit is analogue but not homologous to the subunit γ of F-ATPases (Fig. 2). Two alternative transcript variants, ATP6V1C1 and ATP6V1C2, a and b, that encode different isoforms, have been found for this gene. Regarding the ATP6V1C1, it is always expressed in all tissues, while ATP6V1C2a, b are found in lungs, kidneys and epididymis with an actin-binding function (57).

The C subunit is the 40-kDa protein, located in the V1 domain of the V-ATPase. The novel imaging techniques such as SAXS (small angle X-ray scattering) allow describing the structure and morphology of the C subunit and its involvement in the regulation of V-ATPases (58,59).

By means of immune electronic microscopy, we can determine the spatial location and distribution of the various subunits, in this case of the C subunit (60,61). The model proposed by Zhang *et al* coincides with the one proposed by Drory *et al* which stipulates that the crystal structure of the C subunit consists of two globular domains connected by a flexible connection (62) (Fig. 3).

Inoue and Forgac, besides establishing the crystal structure of the C subunit and its importance in the reversible dissociation as a mechanism for monitoring the V-ATPase activity, describe the connection of the latter with the G and E subunit of the V1 domain and the A subunit of the V0 domain, establishing the importance of the C subunit as the highest responsible for the enzyme control (63,64).

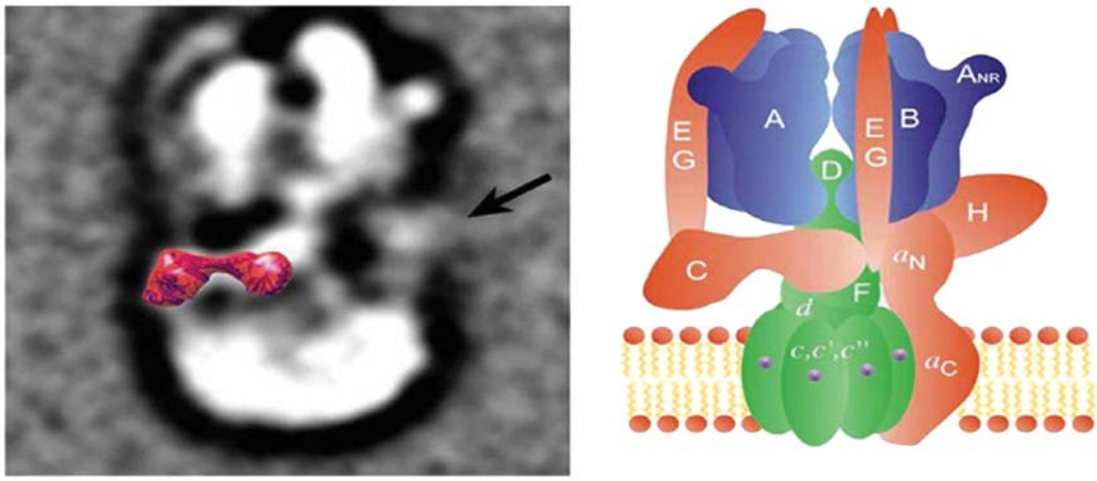

Figure 3. Image of the crystallographic structure of the C subunit and schematic work model. (A) The surface representation of the crystal structure of yeast subunit C is superimposed to match the observed density in the yeast V-ATPase projection. (B) Schematic working model of the subunit arrangement in the yeast V-ATPase. Modified from Zhang *et al* (60).

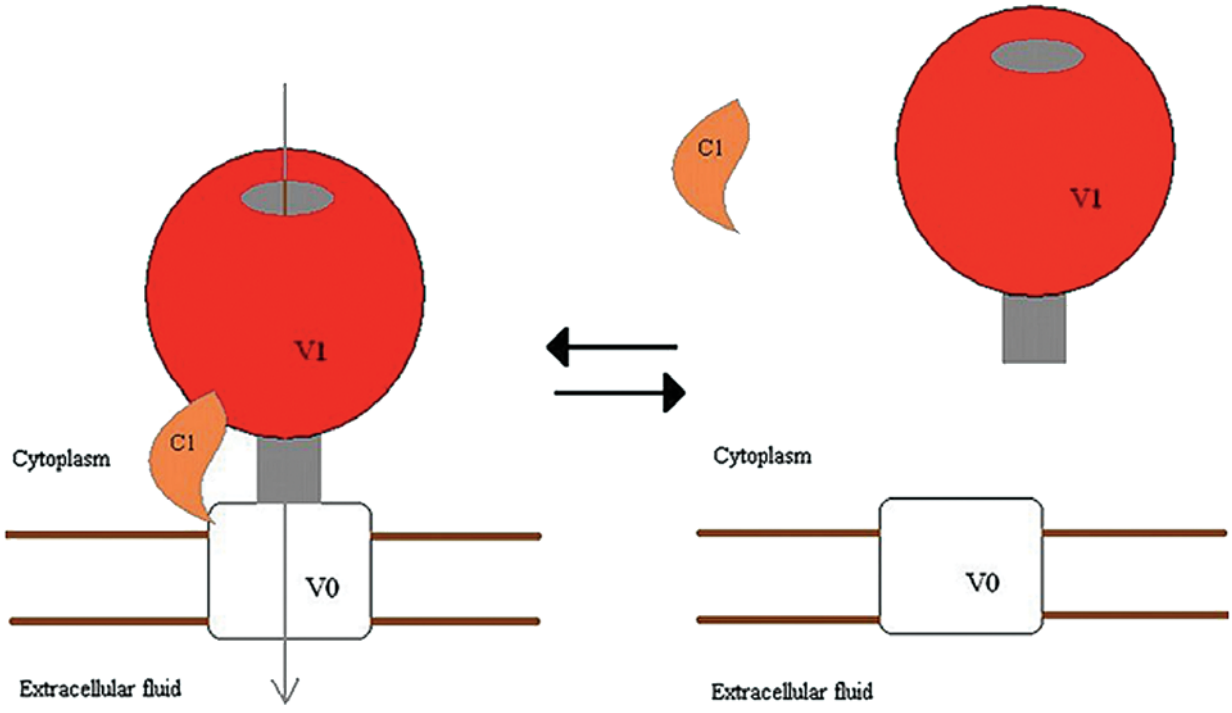

Figure 4. Structural model of the vacuolar H^+-ATPase, comprising a membrane component (V0) and a catalytic cytosolic component (V1). Dissociation of the VATPase into the V1 and V0 components is regulated by the C1 subunit. Obtained from Otero-Rey (31).

In an experimental model, Peng *et al* show that the C subunit is crucial to proton secretion function of the V-ATPases, since the ATP hydrolysis is blocked without it (65).

To many authors, the most important function of the subunit C is the control of reversible dissociation. According to Grüber *et al* subunit C is intimately involved in the reversible dissociation of the V0 and V1 domains. The nucleotide occupation of this latter and the conformational change in its structure allow such a dissociation (66,67).

At first, Puopolo *et al* assert that during the formation of the complex V1V0, subunit C speeds up the process but it is not essential (68), although these results are discussed in other studies of the same period of time, that defend the hypothesis of the C subunit as the only regulatory of the dissociative mechanism (69). This theory is supported by Drory *et al* who state that the C subunit is the only responsible for the *in vivo* dissociation of the V-ATPase (62). According to Voss *et al* the C subunit is responsible for producing the dissociation of the V-ATPase in the cytosolic V1 complex and in the membranous V0 complex through interaction with A-kinase protein. It seems that the C subunit serves as a substrate for the A-kinase protein and its phosphorylation may be the main mechanism of forming the active V1V0 holoenzyme (70).

Another mechanism of reversible dissociation regulated by the C subunit is the separation of V1V0 holoenzyme in V1 and V0 subcomplex, which is carried out through binding this

holoenzyme to the F-actin next to the basement membrane of epithelial cells. It seems that the C subunit acts as an anchor protein, allowing the connection between the V-ATPase and the actinic cytoskeleton (32).

In a study of our research group, we have demonstrated the overexpression of the ATP6V1C1 gene in OSCC biopsies. It seems that the C1 subunit of V-ATPases is responsible for allowing the assembly of membranous V0 component and cytosolic catalytic component (31) (Fig. 4). The RAVE complex (V-ATPase regulator and endosomal membranes) is essential for stable assembly of the C subunit of V-ATPase (64,71).

Smardon and Kane found *in vitro* that without the C subunit, the assembly of the two domains occurred, but the V1V0 complex was highly unstable and the activity of the V-ATPase extremely low, suggesting the exclusivity of the C subunit in regulating the complex V1V0 assembly. In the same way, the C subunit is incapable of getting assembled to the V-ATPase without RAVE and therefore, the enzyme activity is lost (71). These data are supported by the study made by Keenan and Kane who find a 48% higher decrease in catalytic activity, without affecting the enzyme assembly in experimental models with different mutations in the gene of the C subunit (72).

Previous studies suggest that the cells that express high levels of C subunit have an increased resistance to chemotherapeutic agents, so they may be a possible target in anticancer therapy (73).

Murakami *et al* found an overexpression of the ATP6C gene or C subunit in the cisplatin-resistant tumors, a logical fact given the V-ATPase increased number and activity in cases of chemoresistance and the importance of this subunit in the regulation of the pump (74).

5. Conclusions

Cell pH is crucial for several biological functions such as cell proliferation, invasion and metastasis, drug resistance and apoptosis. The hypoxic conditions are frequent phenomena during the development of oral cancer and trigger an intra- and extracellular acidosis. This cellular acidosis seems to be mainly controlled by the V-ATPases, which are clearly involved in cell transformation, carcinogenesis and metastasis.

The inhibition of V-ATPase with PPIS allows the anticancer drugs to enter and act within the tumor cells, causing apoptotic mechanisms that lead to the inhibition of tumor growth.

The involvement of the C subunit of the V1 domain in the enzymatic function of the V-ATPases, highlights the need for further research of specific inhibitors of the above-mentioned subunit in order to control the disastrous consequences of cancer.

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