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THE ROLE OF REDOX HOMEOSTASIS BIOMARKERS IN CLEAR CELL RENAL CELL CARCINOMA DEVELOPMENT AND PROGRESSION

ULOGA BIOMARKERA REDOKS HOMEOSTAZE U NASTANKU I PROGRESIJI SVETLOĆELIJSKOG KARCINOMA BUBREŽNOG PARENHIMA

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Abstract

The clear cell renal cell carcinoma (ccRCC) is the most frequent and the most aggresive subtype of renal cell carcinoma usually detected at an already advanced stage. It might even be observed as a metabolic disease since complex molecular changes and disturbed redox homeostasis are its hallmark. As certain changes are characteristic for tumorigenesis, while some other for metastatic disease, the identification of metabolic modifications could also point out the stage of tumor progression. Hypoxia inducible factor, as a factor regulating transcription of genes encoding glycolytic enzymes, as well as controlling lipid accumulation, has a particular place in ccRCC development. Additionaly, disturbed redox homeostasis induces the Keap1/Nrf2 pathway which further modulates the synthesis of phase-II detoxifying metabolism enzymes. The upregulation of glutathione transferases, Pi class especially, inhibits kinase-dependent apoptosis that is essential in tumor progression. Furthermore, hydrogen peroxide (H2O2) acts as a signaling molecule conveying redox signals, while superoxide dismutase, as well as glutathione peroxidase are enzymes involved in its production and degradation. Hence, the activity of these enzymes impacts hydrogen peroxide levels and consequentially the ability of ccRCC cells to evade negative effect of reactive oxygen species.

Keywords:
clear cell renal cell carcinoma,
redox homeostasis,
hypoxia inducible factor,
glutathione S-transferases,
superoxide dismutase,
glutathione peroxidase

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Sažetak

Svetloćelijski karcinom bubrežnog parenhima (sKBP) predstavlja najčešći i najagresivniji podtip karcinoma bubrežnog parenhima, koji se u najvećem broju slučajeva otkrije u već uznapredovaloj formi. Smatra se da bi ga trebalo posmatrati kao metaboličko oboljenje s obzirom na to da su za njegov nastanak ključne promene u metaboličkim putevima, kao i poremećaj redoks homeostaze. Kako su određene promene karakteristične za nastanak tumora, a druge za metastazni proces, prepoznavanje metaboličkih modifikacija moglo bi da ukaže na stadijum tumorske progresije. Hipoksijom indukovani faktor, kao jedan od faktora koji kontroliše transkripciju gena koji kodiraju enzime uključene u proces glikolize, kao i faktor koji utiče na akumulaciju lipida, imaju značajnu ulogu u nastanku sKBP. Pored toga, poremećena redoks homeostaza indukuje Keap1/Nrf2 signalni put, koji dalje utiče na sintezu enzima uključenih u drugu fazu detoksikacije, uključujući glutation transferaze. Njihovo pojačano prisustvo, naročito Pi klasa, može inhibirati proces programirane smrti ćelija, što je esencijalno za progresiju tumora. I vodonik-peroksid (H_2O_2) ima ulogu u prenosu signala u redoks senzitivnim putevima S obzirom na to da su superoksid dizmutaza i glutation peroksidaza dva enzima uključena u stvaranje i razgradnju H_2O_2, aktivnost ovih enzima značajno utiče na nivo ovog molekula i, posledično, na sposobnost ćelija sKBP da izbegnu negativan efekat reaktivnih kiseoničnih radikala.

Ključne reči:
svetloćelijski karcinom bubrežnog parenhima, redoks homeostaza, hipoksija inducibilni faktor, glutation-S transferaze, superoksid dizmutaza, glutation peroksidaza

Introduction

Malignant cells have several characteristics which separate them from normal tissue cells. Those are the following: the extensive metabolic reprogramming, the synthesis of growth signals, the lack of response to anti-growth signals, the measureless proliferation rate, the evasion of apoptosis, the avoidance of destruction by immune system, the ability to promote angiogenesis and the potential of tissue invasion and metastasis (1, 2).

Since cells are hastily multiplying, tumor masses rapidly increase in size and diameter, distancing from areas of perfusion. Once normal, the environment becomes nutrient-poor and hypoxic. To provide sufficient energy to cells that are proliferating, cellular metabolism must be greatly enhanced. Altered cells with increased basic needs continually consume molecules of necessity while the needed influx remains remoted. Overcoming numerous obstacles is possible only with specific changes in metabolism. Besides, attained alterations have to enable tumor cells to endure potentially harmful environment as well (3).

Although malignancies differ among themselves in their genetic and histological foundation, inevitable modifications of bioenergetics, empowering synthesis of biomolecules and reestablishing favorable redox balance, remain constant (3). Therefore, the transitions of metabolic pathways represent one of the main areas of research interest today (3). As certain processes are fundamental in tumorigenesis and some in late metastatic disease, the identification of metabolic modifications could also point out the stage of tumor progression (3). The effort of finding which metabolic alterations have taken place leads to recognizing vulnerabilities and developing treatment strategies. This broadens potentials for further genetic and molecular explorations enabling better understanding and improvement in management of all sorts of tumors.

The renal cell carcinoma (RCC) comprises almost 90 % of all renal malignancies, while 20 – 40 % of patients diagnosed with this tumor die annually (4, 5). Due to high mortality rate, substantial endeavors in understanding its pathogenesis are being made. Among histologic subtypes defined by World Health Organization (WHO) classification, the clear cell RCC (ccRCC) originating from cells of proximal convoluted tubule, is the most frequent and the most aggressive one when matched for stage and compared to the other subtypes (4). It is usually presented at an already advanced stage with formerly formed metastases (6). Characteristically clear cytoplasm visible on microscopy, as well as accumulation of neutral lipids, rather resembling adipocytes, represent its hallmark, and eosinophilic granular cytoplasm is particularly seen in high grade tumors (6). Furthermore, some imply that pathognomonic adipogenesis helps ccRCC cell survival (7). As in many other tumors, upregulation of aerobic glycolysis and lactic acid fermentation which is called the Warburg effect, take place in higher grades of RCC as well (8). Complex molecular changes in metabolism of glucose, lipid and amino-acids, representing features of this tumor, suggest that it should be analyzed such as a metabolic disease (8). Numerous authors emphasize that classification based on molecular specificities would allow identification of subtypes with negative clinical features (9–11).

The role of hypoxia inducible factor and von Hippel Lindau protein in tumorigenesis

Prevailing hypoxia is one of the most important difficulties that must be mastered by cancer cells. Hypoxic conditions characteristic for malignant tissues lead to selection of cells that are able to adapt and switch to oxygen- and respiratory chain-independent ATP synthesis (12). At the same time, oxygen-scarce surrounding by all means activates pathways oriented towards the repairment of the disturbed homeostasis (13). Hypoxia inducible factor

(HIF) has proven itself to be one of the most important regulator factors in cells' adaptation process and gives answers to numeorus questions involving their potential for growth in hostile milieu.

Within the normal tissues where the supply of oxygen is adequate, oxygen binds to Fe(II) comprising the prolyl-4-hydroxylase domain (PHD) proteins. They enable the hydroxylation of two proline residues within the hypoxia inducible factor-α (HIF-α) subunit. All these steps are necessary for von Hippel-Lindau protein (pVHL) to recognize HIF-α, ubiquitinate these hydroxylated proline residues and label the factor for degradation (14, 15). Therefore, the level of HIF-α is regulated by its constant oxygen-dependent degradation. Hypoxic conditions reveal its further functions which are suppressed in healthy tissues.

Without oxygen binding to the active center of the PHD enzymes, HIF-α evades being recognized by pVHL and marked for consequent proteasome-dependent degradation. Stabilized HIF-α translocates into the nucleus and forms a heterodimer with HIF-β subunits (16). Within the nucleus, HIF heterodimer binds to hypoxia responsive elements (HRE) located on the promoter region of HIF target genes (17). These HREs are essential part of genes encoding erythropoietin and vascular endothelial growth factor (VEGF) that will take part in providing higher oxygen influx (17). Transcription of genes encoding enzymes enabling cells to increase ATP production by glucose uptake is portion of this HRE network as well (15, 17).

However, hypoxic conditions are not the only possible inducer of this set of steps. In the cases of the von Hippel Lindau tumor suppressor gene (*VHL*) mutations, altered pVHL or even the complete lack of it leads to the stabilization of HIF-α just the same as hypoxia does (18). When it comes to von Hippel Lindau disease, biallelic inactivation of VHL is in the core of development of hemangioblastoma, renal cell carcinoma, epididymal cystadenomas and neuroendocrine tumors occurring with high prevalence in this disease (19). Besides occurring as a part of VHL disease, genetic changes in VHL on chromosome 3 are presented in up to 80 % of ccRCC cases (20).

As a heterodimer, HIF is consisted of α and β subunits. The expression of HIF-β is constitutive. On the other hand, HIF-α has three forms HIF-1α, HIF-2α, HIF-3α. While investigating the role of these forms, HIF-1α and HIF-2α were shown to have slightly opposite functions. In the course of earlier investigations, clear cell RCCs were subdivided depending on overexpression of HIF-α forms, where subtype H1H2 has both HIF-1α and HIF-2α equally expressed, while H2 has only HIF-2α and presents with higher proliferation rates than H1H2 (11). It was implied that HIF-1α is involved in initiation of apoptotic pathways in contrast with HIF-2α playing role in regulation of cell growth, proliferation and angiogenesis (21, 22). The third form's activity and involvement in development of cancer still remains unclear (23).

The HIF system as whole regulates transcription of genes encoding enzymes of almost every step in the glycolytic cycle as well as glucose transporters (24). This way it contributes to the Warburg effect dominant in tumor cells. Enzymes involved in glycogen biosynthesis and its storage, are the part of the HIF target genes as well (24). This might represent an adaptive defense mechanism for the possible energy deprivation consequent to the overwhelming proliferation rate. Furthermore, in order to be able to oppose highly conserved endoplasmic reticulum stress response which in hypoxic conditions may cause cellular death, lipid accumulation is controlled by HIF pathway and presents one of ccRCCs important features (7).

Keap1/Nrf2 pathway and cytoprotective response to disturbed redox homeostasis

Concurrently with hypoxia promoting HIF-α stabilization, cellular changes in energy consummation, result into another difference which is essential in carcinogenesis and further progress of the disease. Reactive oxygen species (ROS) are without a doubt one of the game-changers in tumor development and metastatic potential. Some of these reduced metabolites of oxygen – superoxide anion •O2−, hydrogen peroxide H_2O_2, hydroxyl radical •OH, as well as singlet oxygen and alpha oxygen, act as strong oxidizing agents (25). Distinctive disturbance of redox homeostasis causing reactive oxygen species accumulation stimulate the emergence of augmentation of resistance mechanisms which protect cancer cells (26). Broken redox homeostasis and the elevation of ROS activate cytoprotective response. One part of this reaction is the Keap1/Nrf2 (Kelch-like ECH-associated protein 1/ nuclear factor erythroid 2-related factor 2) pathway induction (27). Among many tumors associated with Keap1/Nrf2 alterations, the ccRCC is one of them.

Nuclear factor erythroid 2-related factor 2 is a transcription factor with the ability to protect cells from oxidative damage by recognizing and activating the antioxidant response element (ARE) DNA sequences (28). In the environment with undisturbed redox homeostasis, Nrf2 is bound to Kelch-like ECH-associated protein 1 and suppressed (29). The Keap1 acts as a substrate for specific ubiquitin ligase complex and further leads Nrf2 to its ubiquitylation and degradation (29). It is still unexplained how Nrf2 escapes from the Keap1 dimer and stabilizes in cytoplasm. There are several different mechanisms presented (30–33). However, what remains consistent is that the increase of ROS and electrophiles resulting from the oxidative damage alters the ability of Keap1 to form a dimer and degrade Nrf2 (34). Consequently, the level of Nrf2 is increased within the cell nucleus where it binds to ARE (35). This sequence causes transcription of genes involved in coding phase-II detoxifying enzymes, antioxidants, metabolic enzymes and cellular stress response proteins (29).

At first impression, Nrf2 is a part of mechanism aiming to protect healthy cells from harms caused by ROS and to prevent possible DNA damages which could have oncogenic potential (28). This way, the first, initiating

stage in cancer development is supposed to be blocked by Nrf2 activation. However, with further investigations on tumor progression, it became clear that this transcription factor is somewhat up-regulated within mutated cells, and helps them in forming a defense system of their own (28). Therefore, it might be a target for anticancer therapeutic solutions (27, 28)

With promoter silencing of Keap1 gene, Nrf2 activity is enhanced which predisposes to ccRCC (36). Also, somatic mutations in Nrf2 gene have been reported to take part in development of sporadic ccRCC cases (36). While investigating functional polymorphisms of Nrf2, single nucleotide polymorphism which result in causing self-induction and stronger Nrf2 activation, turned out to be important for further cancer growth among urinary bladder cancer (37). The accumulation of Nrf2 in the nucleus is associated with poor outcome in case of solid tumors, ccRCC as well (36). Similarly, it has been shown that ccRCC patients with genotype corresponding to higher protein expression of Nrf2 have shorter overall survival (38). And in case of response to sunitinib (anticancer drug used to block vascular endothelial growth factor), silencing of Nrf2 in ccRCC model increased sensitivity of cells conducing to apoptosis (39).

Upon entering the nucleus, the binding of Nrf2 to ARE is a part of the adaptational system of the cell to new conditions (36). As previously noted, Nrf2 modulates the transcription of genes encoding phase-II detoxifying metabolism enzymes, as well as phase-III drug transporters (36). One of the main enzymes of interest are glutathione S-transferases (GST) as proteins with roles in numerous catalytic and noncatalytic processes (40). They offer protection from potentially injurious exogenous and endogenous electrophilic molecules by conjugating them with glutathione and thus facilitating their excretion via urine and bile (40).

Among cytosolic classes of GST family, the overexpression of class Pi is attributed to Nrf2 activation (41). Mutations resulting in Keap1/Nrf2 pathway disturbances and the resulting GSTP transcription might be constitutive in cancers resistant to chemotherapeutics (42). However, not only is transcription of GSTP controlled by Nrf2 but this class is also able to influence Nrf2 stabilization and its migration into the nucleus (42). Additionally, GSTP is known to suppress the regulatory mitogen-activated kinase – c-Jun NH2-terminal kinase (JNK1) (42). It is suggested that the possible upregulation of GSTP in patients with ccRCC, further inhibits kinase-dependent apoptosis by forming GSTP1: JNK1/2 complexes (38). What remains to be further investigated is the involvement of Nrf2 to impact this suppression of apoptotic pathways.

The role of hydrogen peroxide metabolism in carcinogenesis

When it comes to direct defense from reactive oxygen species, enzymes involved in hydrogen peroxide synthesis and degradation play an important role in tumorigenesis as well. Mitochondria present continuous producers of basic levels of reactive oxygen species since this is the main localization of cellular respiratory chain (43). Up to 1% of the oxygen entering the respiration cycle is being converted to superoxide anion (44). And while •O2− is not liposoluble because of its charge, hydrogen peroxide is uncharged, stable, and transportable which makes it potent contributor to conveying redox signals (45). As a molecule, H2O2 causes damage of membrane lipids and DNA (43). Still, it takes part in the innate immunity and tissue regeneration (43). Among newly investigated roles, it is found to be a signaling molecule, influencing differentiation, proliferation and migration, as well as activating AMP-activated kinase and supporting glycolysis (45). As much as all these roles may be beneficial for healthy tissue, cells with the oncogenic potential also use H2O2 in their own favor. Besides, the level of oxygen consumption within mitochondria indirectly regulated by H2O2 production and degradation, furthermore stabilizes HIF1α and cause its own activation (46) and the imbalance of superoxide degradation and peroxide synthesis modulate Keap1/Nrf2 pathway (47).

Superoxide dismutase (SOD) acts as an enzyme accelerating further partitioning of $\bullet O2^-$ with H^+ and synthesizing H_2O_2 (45). There are three isoenzymes of SODs, two presented in the cytoplasm (SOD1) and outside of the cell (SOD3) – dismutase with Cu and Zn as metal cofactors, as well as mitochondrial SOD2 with Mn (48). Within cancer cells, the upregulation of SOD2 causes H_2O_2 production which empowers this enzyme to accelerate AMP-activated kinase and promote the Warburg effect (50). In addition, higher concentration of hydrogen peroxide may lead to evasion of apoptosis regulated by tumor necrosis factor-α (51). When it comes to clear cell RCC, it has been suggested that when SOD2 variant unable to be transported into mitochondria is dominant within the cell, its function might be diminished which impacts the risk for this tumor development (38).

Among enzymes decreasing peroxide levels, glutathione peroxidase (GPX) belongs to the family of proteins having important role in H_2O_2 scavenging (52). Eight GPX family members have been identified. The GPX1 is widespread and essential antioxidant enzyme since it catalyzes the extremely fast reduction and transformation of H_2O_2 into neutral H_2O (53,54). It is expected that GPX1 protects cells from ROS induction of cancer transformation and it's activity to be reduced in cancerous kidney (54). However, recent findings suggest that GPX1 is highly expressed in RCC and contributes to higher tumor stage, worse outcome and more invasive metastasis (55). This might target glutathione peroxidase-1 as a protein enabling cancer cells to evade harmful effect of ROS and future biomarker for prognosis of ccRCC patients (55).

Conclusion

Clear cell renal cell carcinoma is an invasive malignancy based on numerous molecular changes which

classify it among metabolic diseases. Gene mutations altering von Hippel-Lindau protein activity and resulting in hypoxia inducible factor stabilization, reveal the importance of hypoxic conditions to ccRCC development. Also, the impact of HIF on glycolytic cycle and lipid accumulation provide adaptation to modified conditions. Additionally, Keap1/Nrf2 pathway plays an important role in protecting mutated renal cells from reactive oxygen species. It even enables cells to evade apoptosis and to be resistant to chemotherapeutics. As a part of extremely complex network of enzymes controlled via Nrf2, glutathione S-transferase class Pi and superoxide dismutase-2 further impact ccRCC ability to form metastasis. Therefore, the understanding of all these pathways and enlightening regulation of enzymes involved in redox homeostasis might contribute to in the right assessment of ccRCC patients and formulation of the most efficient approach in management of the disease.

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