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Can hypoxia marker carbonic anhydrase IX serve as a potential new diagnostic marker and therapeutic target of non-small cell lung cancer?

Review

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Lung cancer represents the leading cause of cancer-related deaths. Non-small cell lung cancer (NSCLC), the most common form of lung cancer, is a molecularly heterogeneous disease with intratumoral heterogeneity and a significant mutational burden associated with clinical outcome. Tumor microenvironment (TME) plays a fundamental role in the initiation and progression of primary *de novo* lung cancer and significantly influences the response of tumor cells to therapy. Hypoxia, an integral part of the tumor microenvironment and a serious clinical phenomenon, is associated with increased genetic instability and a more aggressive phenotype of NSCLC, which correlates with the risk of metastasis. Low oxygen concentration influences all components of TME including the immune microenvironment. Hypoxia-inducible pathway activated in response to low oxygen supply mediates the expression of genes important for the adaptation of tumor cells to microenvironmental changes. A highly active transmembrane hypoxia-induced metalloenzyme – carbonic anhydrase IX (CAIX), as a part of transport metabolon, contributes to the maintenance of intracellular pH within physiological values and to the acidification of the extracellular space. CAIX supports cell migration and invasion and plays an important role in NSCLC tumor tissue and pleural effusion. Due to its high expression, it also represents a potential diagnostic differential biomarker and therapeutic target in NSCLC. To test new potential targeted therapeutic compounds, suitable models are required that more faithfully simulate tumor tissue, TME components, and spatial architecture.

Key words: carbonic anhydrase IX; hypoxia; hypoxia inducible factor 1; tumor microenvironment; spheroid; non-small cell lung cancer

Lung diseases include the whole spectrum of diseases and disorders that prevent the proper functioning of the lungs, affect the respiratory function or the ability to breathe. Mortality from respiratory diseases is the third leading cause of death in EU countries, accounting for 8% of all deaths in 2015 [https://ec.europa.eu/eurostat/web/metadata]. There are three main types of lung diseases: diseases of the airways, diseases of the lung tissue, and diseases of the pulmonary circulation. The most common lung diseases include asthma, lung cancer, inflammatory lung diseases, tuberculosis, chronic obstructive pulmonary disease, and idiopathic pulmonary fibrosis.

Lung cancer was a very rare disease in the past. Currently, it is among the malignant diseases that have one of the highest mortality rates in the world. It is the leading cause of death in men and the second leading cause of death in women after breast cancer [1]. The disease is mostly diagnosed at an advanced stage and only approximately 15% of patients survive more than 5 years. The most common incidence of lung cancer is between 55 and 80 years of age. The most significant risk factors are smoking, secondhand smoke, exposure to radon, asbestos, ionizing radiation, and chronic infections [2]. From the point of biological properties and treatment procedures, we can divide lung cancer into small

cell lung cancer (SCLC), which represents about 15%, and non-small cell lung cancer (NSCLC), which represents about 85% [3].

Non-small cell lung carcinoma

NSCLC is a molecularly heterogeneous disease and, like most malignancies, consists of a subpopulation of cells or clones with different molecular features, leading to intratumoral heterogeneity and thus to metastasis and disease progression. The disease is diagnosed in most cases at an advanced stage.

New clinical studies, immunohistochemical, molecular and genetic analyses with the aim of personalizing the treatment strategy in patients in the initial as well as in the advanced stage of the disease have revealed the need to improve morphological diagnostics. According to the World Health Organization (WHO, 2017), the morphological classification is used as a standard.

The main types of NSCLC include squamous cell carcinoma (30%), adenocarcinoma (40%), large cell carcinoma (15%), but also neuroendocrine carcinoma (NET), adenosquamous and sarcomatoid carcinoma. In addition to these types, WHO lists other subtypes [4].

Among the most frequently represented are lung adenocarcinomas, which occur more often in ex-smokers and non-smokers, and squamous cell lung cancer, which occurs more often in smokers. The great variety of histopathological types reflects the heterogeneity of lung cancer, which affects the pathological diagnosis, pathogenesis, prognosis of the disease but also the therapeutic decision [5]. Therefore, the goal is to obtain a detailed histological diagnosis and choose the most appropriate therapeutic strategy based on the mutational profile. Cancer staging is done according to the International Association for the Study of Lung Cancer (IASLC) TNM classification-8th Edition.

Mutations associated with NSCLC. Mutations of epidermal growth factor receptor (EGFR) belong among often-analyzed mutations. The most frequent mutations are exon 19 deletion and L858R in exon 21, which represent approximately 90% of mutations in NSCLC and lead to a high sensitivity to tyrosine kinase inhibitors (TKI) [6]. Rare mutations form the remaining 10% of EGFR mutations in NSCLC. Substitutional mutations G719X in exon 18, L861Q in exon 21, S768I in exon 20, and exon 20 insertion belong among the most frequent ones. At present, there is no approved therapy targeting such rare mutations, hence chemotherapy often presents the only possible therapy for such patients [7].

Furthermore, mutations in anaplastic lymphoma kinase (ALK), which induces cell growth, are detected and analyzed. Mismatch of the ALK gene occurs in approximately 3–7% of all NSCLC cases. The most frequent fusion partner of ALK is EML4 (echinoderm microtubule-associated protein like 4) whilst this fusion results in significantly increased activity of ALK and in the origin and progression of oncological disease [8]. Tyrosine kinase inhibitors targeting ALK prolong the survival of patients with advanced ALK-positive NSCLC.

Genomic mismatches were also described for the proto-oncogene ROS1 tyrosine kinase receptor. The overall prevalence of ROS1 fusions for NSCLC is 2–5%. ROS1 belongs to the subfamily of tyrosine kinase insulin receptors, however, its precise function has not been elucidated yet. Gene fusions with several fusion partners are known, among them being e.g. CD74, ezrin, syndecan, tropomyosin, and many others [9].

NSCLC carrying mutations in the human epidermal growth factor receptor 2 (HER2) is considered a different molecular subtype. In NSCLC, HER2 is activated by gene mutations (1–4%), amplifications (2–5%), and protein overexpression (2–30%), with different prognostic and predictive results [10]. Exon 20 insertions influencing the kinase domain are the most frequent HER2 mutations (96%). They are similar to the activating mutations of EGFR (other than T790M), which are connected with resistance to the first as well as second generation of TKI.

cMET oncogene, also known as tyrosine protein kinase MET or hepatocyte growth factor (HGF) receptor, is predominantly expressed in epithelial cells and plays an important role in embryogenesis, tumor growth, and metastasizing. Dysregulation of the MET/HGF pathway contributes to the proliferation, invasion, and metastasizing of tumor cells. Changes in cMET in NSCLC include single mutations, amplification, fusion, and overexpression of the protein, and are linked with a poor prognosis. Single mutations, deletions, insertions, and complex mutations of the exon 14 lead to reduced degradation of the MET receptor, which causes the activation of MET signaling and tumorigenesis [11]. cMET amplification, leading to the activation of tumorigenesis, was reported for 2–5% of NSCLC [12].

KRAS mutations belong among the most common factors influencing NSCLC. They occur in approximately 25–30% of lung adenocarcinoma, they rarely occur in squamocellular carcinoma. More than 80% of KRAS mutations can be found in codon 12, the most frequent being KRAS G12C (40%), KRAS G12V (18–21%), and KRAS G12D (17–18%). Unlike other mutation types, KRAS mutations are usually connected with smoking [13].

Tumor microenvironment

Cells in tissues constantly perceive the biochemical and mechanical state of their microenvironment, convert extracellular signals into intracellular ones, integrate these signals, and adequately respond to them. Responses at the cellular level may include changes in metabolism, gene expression, growth, differentiation, cell division or apoptosis. Any disruption of these processes can lead to abnormal, uncontrollable tissue growth-the formation of a tumor but also to other changes, for example, to the development of inflammation

or metabolic diseases [14]. The tumor microenvironment (TME) plays a central role in the initiation and progression of primary *de novo* lung cancer [15–17]. In addition, extrathoracic localized malignancies (e.g., melanoma, colorectal, and breast cancer) are capable of reprogramming the lung microenvironment in favor of the colonization and growth of disseminated tumor cells with the aim of forming secondary lung tumors [18].

TME composition. The TME is very heterogeneous and characterized by dynamic interactions between its individual components. It plays a fundamental role in the process of tumorigenesis and significantly influences the response of tumor cells to treatment [14]. Tumor cells interact with surrounding cells through the blood and lymphatic system and thereby influence tumor development and progression. The TME is composed of tumor and non-tumor components that play a critical role in all stages of tumorigenesis, interacting with each other, stimulating and facilitating uncontrolled cell proliferation (Figure 1). In contrast, tumor cells invade healthy tissues and spread to other parts of the body via the lymphatic or circulatory system [19]. From a histological point of view, tumor stroma and tumor parenchyma (tumor's own cells) participate in tumor formation. The tumor stroma is represented by blood vessels, supporting tissues, fibroblasts, and immune cells. It serves as a framework for the organization of the parenchyma but also participates in ensuring nutrition. It has a transport role in signal transduction, is a reservoir of growth factors, and plays a key role in angiogenesis and metastasis.

Hypoxia and hypoxia-induced acidosis trigger signaling pathways leading to the adaptation of tumor cells to altered conditions in the TME. Long-term hypoxia in tumor tissue results in cell death and the formation of necrotic areas surrounded by surviving adaptive cells. Hypoxia, as a component of the lung TME, promotes the conversion of normal lung fibroblasts into cancer-associated fibroblasts [20]. Physical and chemical factors of the TME represented by hypoxia and associated acidosis, impair the function of natural killer cells and cause a decrease in their cytotoxicity. In NSCLC, a high activity of HIF1 transcription factor is associated with a negative impact on overall survival [21]. During hypoxia, extracellular acidosis and increased adenosine levels can block NK cell activation, proliferation, and cytotoxicity [22].

Due to the rapid proliferation of tumor cells, reprogramming of cell metabolism occurs in tumors. As a result of oncogenic metabolism, a number of "acidic" metabolites are produced in the cell. In order to maintain pH homeostasis, it is therefore necessary to remove these products from the cytosol to the external environment.

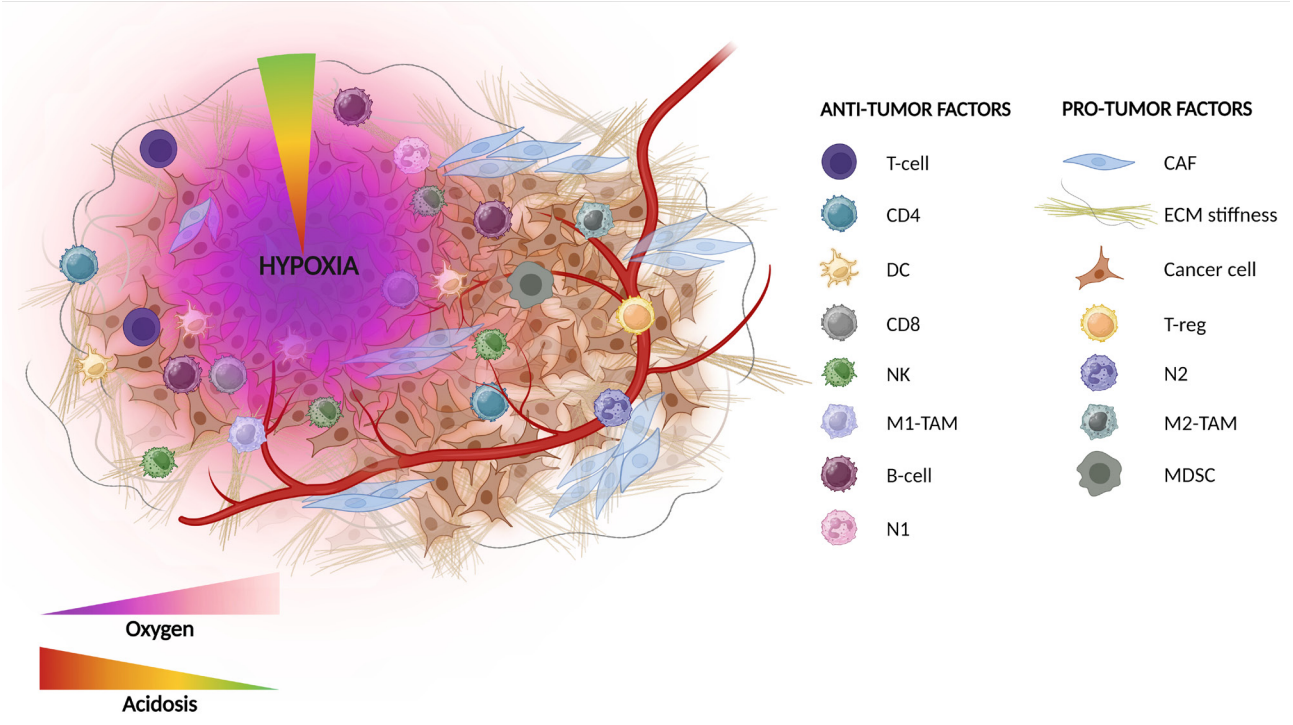

Figure 1. Heterogeneity of tumor microenvironment (TME). The integral components of the microenvironment of fast-growing solid tumors are gradients of oxygen and pH, resulting in the origin of the areas with hypoxia and acidosis. Besides tumor cells themselves, the TME contains a plethora of non-tumor cell types, such as fibroblasts, endothelial, and immune cells, with anti-tumor and pro-tumor activities. Proteins of the extracellular matrix are also an integral part of the TME.

Acidosis represents a strong selection pressure that contributes to the emergence of aggressive, therapeutically resistant clones [23]. At the genomic level, acidosis can increase genome instability through chromosome breaks or translocations [24]. Tumor cells have developed mechanisms to adapt to this environment or to maintain intracellular pH homeostasis [25, 26]. Hypoxia is usually associated with acidosis, but acidosis can also occur in the TME under normoxic conditions due to the preference of tumor cells to use glycolysis [25, 26]. In addition to lactate, a significant source of the acidic extracellular environment in the TME is also CO_2, which is a product of many metabolic processes of tumor cells, such as oxidative metabolism or the pentose pathway [27].

Carbonic anhydrase IX-its role in tumor cells and in the formation of TME. Carbonic anhydrase IX (CAIX) belongs to the family of zinc-binding metalloenzymes. This hypoxia-induced enzyme with an extracellular catalytic domain plays an important role in the maintenance of intracellular and extracellular pH homeostasis and by its enzymatic activity significantly affects the TME.

There are 15 human isoforms of carbonic anhydrases out of which three are inactive and the activity of the remaining 12 isoforms varies from weak to very strong. The majority of these isoenzymes are expressed in differentiated cells where they fulfill specialized tasks in various tissues and organs, especially in the metabolically highly active ones, such as the brain, kidney, stomach, pancreas, etc. [28]. CAIX is the only isoenzyme, which is associated mainly with tumors and its expression in healthy tissues is limited to the mucosa of stomach and colon crypts. CAIX was found in a wide range of solid tumors and its presence is often linked with an aggressive tumor phenotype, which stems from its strong hypoxic regulation at several levels [29, 30].

Regulation of CAIX expression. Hypoxia, as an integral part of the TME, is a feature of most solid tumors. Oxygen concentration in normal human lung tissue is approximately 5.6% O_2, whereas in NSCLC tumors it is between 1.9–2.2% [31]. Hypoxia occurs as a result of the rapid proliferation of tumor cells, which reach a distance exceeding the diffusion capacity of oxygen from the nearest blood vessel (100–150 μm) and are insufficiently supplied with oxygen and nutrients [32]. Hypoxic areas in tumor tissue are associated with increased genetic instability and a more aggressive phenotype, which correlates with the risk of metastasis. According to its response to hypoxia, CAIX is among the most strongly hypoxically regulated genes. The main reason lies in the fact that the *CA9* gene is transcriptionally regulated by hypoxia via HIF1 binding to the HRE (hypoxia response element) sequence localized directly before the transcription starting point [33]. Therefore, HRE is an important part of the basic promotor of the *CA9* gene and, together with a neighboring binding site for SP1, the module SP1-HRE acts as a main mediator of *CA9* transcription [34]. *CA9* promotor contains activatory cis-elements including HIF1 binding HRE (–10/–3), ETS-1 binding site (–16/–12), SP1 binding regions (–45/–24 and –163/–145), AP2 and AP1 binding regions (–74/–56 and –101/–85) and many others the function of which in *CA9* regulation has not been elucidated so far. Transcription factors ETS-1, AP1, and AP2 perform a modulatory role in various tumor cells [34–36]. There is also a negative regulatory region (–134/–110), which binds a transcription repressor identified as MORC2 complex (microorchidia/HDAC2) which causes decreased acetylation of *CA9* promotor [37].

CA9 gene is located at chromosome 9p12-13, consisting of 11 exons and 10 introns [38]. The exon 1 codes N-terminal domain homologous with proteoglycans (PG domain), exons 2–8 code a carbonic anhydrase catalytic domain (CA), exon 9 corresponds to the aminoacid region preceding the transmembrane region (TM) coded by the exon 10. The exon 11 codes the intracellular part, i.e., IC tail, and 3′-untranslated region (3′-UTR) containing one AU-rich region known as an unstable motif [38, 39].

SP1 is involved in inducing the *CA9* transcription under the conditions of high cellular density and probably also by acidosis (in normoxia and hypoxia) in a cell type-specific manner, which seems to depend on a threshold capacity to regulate pH, specific for each cell type [34]. As the expression and activation of HIF are influenced by oncogenic signaling, *CA9* transcription is also increased in response to the activation of MAPK and PI3K pathways and of "upstream" kinases, including Src oncoprotein and EGFR [40, 41]. *CA9* is subjected to alternative splicing resulting in two products: a shorter one missing exons 8 and 9, hence without catalytic and transmembrane domains, which is not located at the cell membrane, and a longer one containing all 11 exons. The function of a shorter variant has not been elucidated yet [42].

CA9 mRNA is stabilized by hypoxia-activated cytoplasmic accumulation of beta-catenin, which binds to 3′-UTR of *CA9* mRNA with the assistance of mRNA binding protein HuR and facilitates the transport of HuR/mRNA complex into the ribosomal subunit 40S [43].

Basic characteristics of CAIX. CAIX is a transmembrane protein consisting of 459 amino acids with a molecular weight of 54/58 kDa. It comprises a large extracellular domain (ectodomain ECD), a transmembrane part (TM), and a short intracellular tail (IC). ECD domain contains a central catalytic domain (CA) and N-terminal proteoglycan domain (PG), which exhibits 38% homology with a binding domain for keratan sulfate in the large proteoglycan aggrecan [38, 44]. A comparative analysis of the key sequence of the PG domain (amino acids 62–93) containing a repetitive pentameric motif EEDLP showed a 50% match with the aggrecan region with a conserved hexapeptide sequence, which is modified by keratan sulfates and able to bind collagen and hyaluronan [45, 46]. Recently, a direct binding between CAIX and collagen was proved by surface plasmon resonance [47]. PG is unique for CAIX, this domain is not present in other isoforms of carbonic anhydrases. The central

CA part includes an active site, with three conserved histidines binding zinc ions and the fourth histidine serving as a proton exchanger. This domain exhibits enzymatic activity, ensuring a reversible conversion of CO_2 to H_2CO_3, which spontaneously dissociates to HCO_3^- and H^+ [44, 48]. The intracellular tail (IC) is responsible for the correct localization of the protein in the cell membrane, which is a prerequisite for crucial extracellular functions of CAIX [49]. At the functional level, CAIX is activated by a hypoxia-induced increase of cAMP level and a subsequent activation of protein kinase A (PKA) that phosphorylates Thr443 in the IC [50]. Simko et al. (2020) described a new role of the PIMT protein, which interacts with a methyl group in the amino acid alanine in the IC of CAIX, whilst a disruption of this interaction led to perturbations in CAIX-mediated pH regulation [51]. This finding indicates a possible new mechanism of the regulation of CAIX enzymatic activity.

Two sites of glycan attachment and one site of GAG conjugation were identified in the CAIX molecule. O-glycosylation site is at Thr115 between PG and CA domains, a small di-, tri-, or tetra- saccharide structure binds here [44]. Asn346 in the CA domain is glycosylated by N-type high-mannose oligosaccharide. Christianson et al. (2017) identified Ser54 in the PG domain as a site of the conjugation of heparan sulfate or chondroitin sulfate. This high-molecular form of CAIX was denoted as a hypoxia-regulated part-time proteoglycan because it can exist in forms with or without GAG modification [52]. The extent of GAG modification varies, whilst this modification is involved in adhesion, migration, and influences signaling pathways, disrupts metabolon formation, and reduces acidification ability. Reduced GAG modification of CAIX was observed in the inflammatory environment in hypoxia [47].

The level of membrane-localized CAIX is also affected by the shedding of its extracellular part, also called ectodomain (ECD) [53]. The process of shedding creates soluble molecules from ectodomains of cell surface proteins, significantly influencing biological functions of these proteins, and enabling their autocrine or paracrine action. ECD shedding is low in normal conditions, but it is dramatically increased after its activation by ionophores, growth factors, cytokines, stress factors, and some chemotherapeutical agents. Shedding of ECD is performed by metalloprotease ADAM17 (a disintegrin and metalloproteinase), which is upregulated under strict hypoxia, and deletion of amino acids 393–402 in CAIX cancels this process [54]. Results obtained in C33a cells expressing a CAIX variant unable to be shed show changes in the extracellular proteome, and increased migration and invasion *in vitro* in comparison with cells expressing wild-type CAIX. *In vivo*, they exhibit faster growth in xenografts and increased metastasizing into the lungs [54]. CAIX ECD shedding can also be mediated by ADAM10, which facilitates shedding when ADAM17 is not present [55].

Biological functions of CAIX in cancer cells. CAIX is among the enzymes, which significantly contribute to the adaptation of tumor cells to changing TME. Hypoxia induced CAIX expression and its activity in maintaining acid-base balance predetermine the importance of this protein in the processes leading to the management of microenvironmental stress [56]. Clinical data based on the immunohistochemical staining of CAIX protein in various tumor types show that a high level of CAIX is often linked with cancer progression and a poor prognosis.

Due to hypoxia, the metabolism of cancer cells is shifted from oxidative phosphorylation to anaerobic glycolysis. This shift is accompanied by the acidification of the intracellular environment, which must be kept within a relatively narrow pH range.

By its catalytic function in collaboration with bicarbonate transporters (Figure 2), CAIX contributes to pHi regulation, parallelly generating protons, which acidify the extracellular environment. Slightly alkaline pHi provides a proliferative advantage as it enables biosynthetic reactions, whilst acidic pHe induces cell migration and activates proteases degrading extracellular matrix, which supports the invasion of tumor cells into the surrounding tissues [57]. CAIX thus protects tumor cells from intracellular acidosis, which originates due to the excess of lactate, protons, and CO_2 generated by oncogenic metabolism [58]. Besides that, CAIX facilitates cell migration and invasion helping tumor cells to escape from hostile hypoxic and/or acidic microenvironment [45, 59, 60]. Studies also showed that the expression and function of CAIX are linked with the resistance of cancer cells to antitumor therapy [61]. Svastova et al. (2003) proved the involvement of CAIX in the regulation of cell-cell adhesion by its interaction with beta-catenin and a subsequent dissociation of E-cadherin from the adhesion complex. Ectopic expression of CAIX in MDCK cells caused the weakening of cell-cell contacts and led to the increased dissociation of cells, facilitating migration [62].

Migration and invasion of tumor cells considerably contribute to metastasis formation. Tumor hypoxia supports epithelial mesenchymal transition, and invasion as was reported by several studies [60, 63, 64]. Via its catalytic activity, CAIX plays an important role in cell migration [59]. CAIX forms a transport metabolon with bicarbonate transporters in the lamellipodia of migrating cells, increasing the flow of ions through the cell membrane and contributing to the creation of a pH gradient along a translocation axis, which is typical for migrating cells with a more acidic pericellular pH at the front part of a moving cell, whilst pHi values have an opposite layout [59].

CAIX is also involved in the focal adhesion process, which is necessary for the migration and invasion of tumor cells during their metastatic spreading. During the migration cycle cells have to repeatedly form and dissolve focal contacts, which have a short life span and a fast cycle of their turnover [66]. Focal contact formation is a process requiring a gradual accumulation of several proteins, which connect integrins and other ECM receptors with the actin cytoskeleton. Integ-

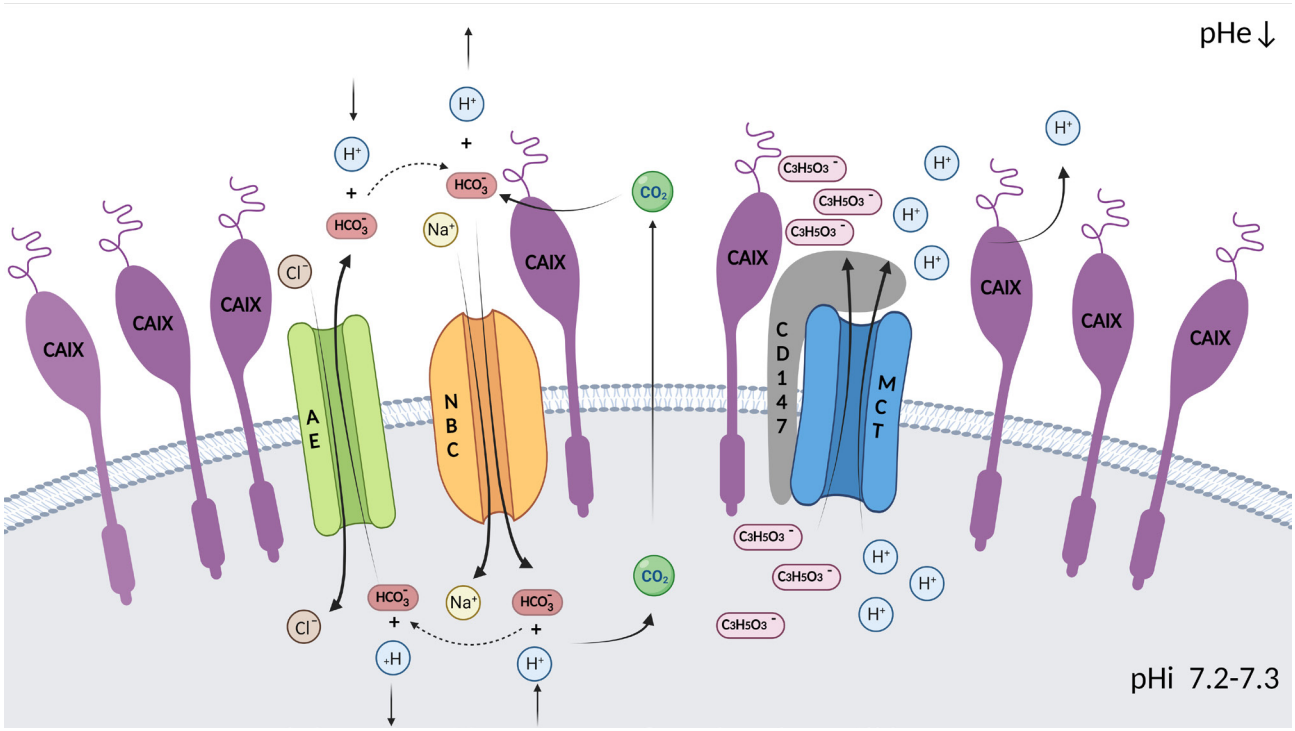

Figure 2. Involvement of CAIX in the transport metabolons facilitating the regulation of pH in tumor cells. By its catalytic activity, CAIX produces protons, which remain in the extracellular space, and bicarbonate anions, which can be transported into the cytoplasm by anion exchangers (AE) or by bicarbonate transporters (NBC) where they contribute to pHi alkalinization. In a non-catalytic way, CAIX facilitates a proton-coupled lactate ($C3H5O3^-$) transport activity of monocarboxylate transporters (MCT). CAIX does not bind to MCT directly, instead, it binds to their chaperone CD147. The PG domain of CAIX acts as a proton antenna, transferring protons through its glutamate and aspartate residues into the surrounding space, thus promoting MCT activity.

rins bind adaptation and signaling proteins, such as paxillin, vinculin, talin, focal adhesion kinase (FAK), Rho GTPases, etc. [66]. Focal contacts grow and dissolve in close relation to actin polymerization and myosin II generated tension [67]. A key molecule for focal contact assembly is the adaptor protein paxillin, which directly binds to integrins [68]. CAIX was detected in nascent adhesions in the lamellipodia of migrating cells where it colocalizes with focal adhesion marker-paxillin [45]. CAIX participates in the regulation of their turnover by its pH regulation function as local alkaline pHi is crucial for actin rearrangement through proteins acting as pH sensors. Csaderova et al. (2013) confirmed the involvement of CAIX in the process of spreading non-migrating quiescent cells where CAIX colocalizes with paxillin in focal adhesions. The experiment with the antibody targeting the PG domain of CAIX and with transfectants expressing CAIX with deleted PG domain indicated a role of the PG domain in this process [45].

The expansion of cancer cells from primary tumors is associated with extracellular matrix (ECM) degradation and with dynamic interactions between ECM and cell cytoskeleton. Actively migrating and invading tumor cells possess small plasma membrane extensions, called invadopodia, at their front side [69]. Extracellular acidification is necessary for invadopodial function through the activation of matrix metalloproteases (MMPs) [70]. pH regulators generating a gradient of pHe and pHi are decisive for the invadopodia function and for tumor invasion. By its enzymatic activity, CAIX contributes to the acidification of the extracellular space and creates a proinvasive TME [59, 71, 72]. Invadopodial functions depend on the Na^+/H^+ exchanger (NHE1) [73], which controls local intracellular alkalinization and extracellular acidification during invadopodia maturation, supporting ECM proteolysis by enhancing the activity of MMPs and cathepsins, which cleave ECM [70, 74]. Local intracellular alkalinization at the invadopodia ends is crucial for actin remodeling via pH-sensitive proteins, such as talin and cofilin, and for subsequent growth of invadopodia [75]. A study by Debreova et al. (2019) demonstrates the presence of CAIX directly in the invadopodia together with the active form of PKA that activates CAIX by phosphorylating Thr443 [76]. At the same time, this phosphorylation is required for the interaction between CAIX and MMP14 during which protons produced by CAIX enzymatic activity are important for the collagen degradation mediated by MMP14 [60].

Debreova et al. (2019) observed reduced metastasis formation in *in vivo* murine model of lung colonization and also in the model of tumor cells' penetration into the chorioallantoic

membrane of a quail embryo after treatment of tumor cells by specific antibodies against PG or CA domain of CAIX, thus confirming the role of both CAIX domains in the tumor invasion [76].

CAIX as a part of the transport metabolon/interactome in the TME. One of the key processes for the survival of tumor cells is maintaining the pH balance. In healthy tissue, the intracellular pH is around neutral, while the extracellular pH is slightly alkaline. In the TME, on the other hand, pHi becomes more alkaline and pHe more acidic [77] due to the accumulation of acidic products of metabolism. Tumor cells are characterized by a reverse pH gradient, i.e. lower pHe (5.8–6.8) and higher pHi (7.2–7.5). The reverse pH gradient brings several advantages – it activates proliferation, invasiveness, metastasis, and inhibits apoptosis [58, 78].

CAIX interactome influencing pH regulation. Along with intracellular and extracellular carbonic anhydrases (CAs), various membrane transporters and exchangers such as NHE, NBC, AE, and MCT are also involved in pH regulation. These form a protein complex known as the "transport metabolon". A transport metabolon is defined as a transient, structurally functional, supramolecular complex of consecutive metabolic enzymes and cellular structural elements in which metabolites are transferred from one active site to another [79–81].

Interaction of CAIX with bicarbonate transporters. The transmembrane localization of CAIX with its extracellularly oriented catalytic domain suggests its spatial and functional association with ion transporters that regulate the local pH gradient. The functional cooperation of CAIX with bicarbonate transporters, specifically with the Na^+/HCO_3^- transporter NBCe1 (SLC4A4) and with the anion exchanger AE2 (SLC4A2) in a protein complex called the bicarbonate transport metabolon has been demonstrated [82]. Svastova et al. (2012) confirmed the direct interaction of CAIX with AE2 and NBCe1 in the lamellipodia of migrating cells through the PLA method (Proximity Ligation Assay), which enables the detection of stable and temporary protein-protein interactions *in situ*. Through the competitive ELISA method, a direct interaction of carnosine with the catalytic domain of CAIX was detected, resulting in a change in the conformation of CAIX, which may affect the interaction with AE2 and NBCe1 [59]. Impaired formation of the bicarbonate transport metabolon was indeed confirmed by a reduced signal reflecting the CAIX-AE2 interaction using the PLA method [83].

During CO_2 hydration catalyzed by CAIX, in addition to HCO_3^- ions, an excess of extracellular protons is generated. It is known that CAIX can interact with NBC1, which carries out Na^+/HCO_3^- cotransport. In the intracellular space, bicarbonate consumes protons by producing CO_2, which diffuses across the membrane and remains in the pericellular space. It has been demonstrated that disruption of pH homeostasis by blocking HCO_3^- import could lead to amelioration of the common resistance of hypoxic tumors to antitumor therapy [84]. Hypoxia-induced CAIX can also interact with NHE1 and also with the NCX1 exchanger. CAIX can facilitate proton export through NHE1 in exchange for Na^+ import, which is likely to occur through a non-catalytic mechanism where CAIX enables intramolecular transport of H^+ through His200, which is independent of CO_2 hydration [85]. An increase in intracellular Na^+ promotes NCX1 reverse-mode activity, in which Na^+ export and Ca^{2+} import occur, removing cytosolic Na^+ in exchange for Ca^{2+} [86].

Interaction of CAIX with monocarboxylate transporters. During hypoxia, there is an increased export of lactate along with protons in tumor cells via the monocarboxylate transporters MCT1 and MCT4 [87]. In hypoxic tumor cells, MCT transport activity may be facilitated through interaction with CAIX. CAIX does not bind directly to the transporter, but to the first globular domain of the MCT chaperone CD147 of the MCT1 and MCT4 transporters [88]. Proton transfer between MCT and CAIX appears to occur through the PG domain of CAIX, which contains 18 glutamate and 8 aspartate residues that could function as a proton antenna for this complex [89], while His200 in the catalytic CAIX domain facilitates the transfer of protons from the catalytic center to the surrounding space and thus supports MCT activity [85]. A study by Ames et al. (2020) pointed to the formation of the transporter metabolon CAIX and MCT1/4 in human breast tumor tissue, but not in healthy breast tissue. The number of transporter metabolons detected by the PLA method increased with increasing tumor stage, and the correlation of CAIX, MCT1, and MCT4 expression with tumor stage and poor prognosis was demonstrated [89].

Interactions of CAIX facilitating tumor cell invasion. Swayampakula (2017) and co-authors showed that CAIX interacts with MMP14 in invadopodia. MMP14 is a proteolytic enzyme degrading the ECM and its activation is important for invadopodial function. The association of CAIX with MMP14 provides protons to activate MMP14, thereby aiding its function in degrading the surrounding matrix, allowing invadopodia to grow [60]. Increased activity of MMP14 in association with intracellular alkalinization inside invadopodia aids in actin elongation and thus in tumor cell invasion [90].

Destabilization of cell-cell contacts plays an important role in tumor progression, facilitating invasion and metastasis [91]. Solid tumors often show impaired expression and/or function of the central cell adhesion molecules E-cadherin and beta-catenin, which are critical for maintaining tissue integrity by generating tight cell-to-cell contacts associated with the cytoskeleton [91]. The polarized distribution of CAIX in normal epithelial cells *in vivo*, its proposed role in cell adhesion and increased expression in tumors led to the discovery of the relationship between CAIX and E-cadherin with respect to subcellular localization and functional interplay [62].

PIMT as a new interaction partner of CAIX. Recently discovered interaction partner of CAIX is the protein PIMT (protein-L-isoaspartyl methyltransferase), whose interac-

tion was found through proteomic analysis and subsequently proven by immunoprecipitation [51]. PIMT is well known for its protein repair function, it regulates e.g. calcium homeostasis by repairing the calcium-binding protein calmodulin, thereby restoring its functions [92], and prevents stress-induced damage by repairing various antioxidant proteins, helping cells avoid apoptosis. The antiapoptotic role of PIMT was studied in neuroblastoma cells, where PIMT protein inhibited the activation of caspase 3 and caspase 9 [93]. The intracellular interaction of CAIX with the cytoplasmic protein PIMT takes place through Ala459, which is the last amino acid of the IC domain of CAIX. This interaction between the C-terminal Ala459 of the CAIX protein and PIMT affects the catalytic function of CAIX, as the structural change of Ala459 disrupts the ability of CAIX to regulate pH, reducing its enzymatic activity and cell migration [51].

Interactions of CAIX with amino acid transporters. A comprehensive study focused on the identification of the protein interactome of CAIX using proximity-dependent biotin identification (BioID) identified more than 140 proteins that interact with CAIX with high probability [60]. In this study, several new potential interaction partners were identified between amino acid (AA) transporters, namely neutral amino acid transporter 1 (LAT1) and glutamine transporters ASCT2 and SNAT2 [61]. The association of CAIX with the glutamine transporter SNAT2 [60], together with the interaction with the essential amino acid transporter LAT1, suggests an active role for CAIX in coordinating the regulation of AA efflux especially since the import of essential AAs such as leucine is coupled to glutamine efflux [94]. Overexpression of the L-type amino acid transporter (LAT1) is a negative prognostic indicator in many types of cancer, and its activity is essential for tumor growth under conditions of hypoxia and lack of nutrients, i.e., conditions in which CAIX expression is induced.

While the metabolic reprogramming of tumor cells leads to the use of glucose as an energy source, it is now well known that tumor cells are able to utilize a diverse spectrum of nutrients, especially glutamine, to support their metabolic and biosynthetic functions [95]. Glutaminolysis metabolically contributes to intracellular CO_2 production through decarboxylation of metabolic intermediates [96]. Thus, the interaction of CAIX with glutamine transporters such as ASCT2 and SNAT2 potentially links glutamine import and metabolism to the efficient control of CO_2 production.

Alanine Serine Cysteine Transporter 2 (ASCT2) or SLC1A5 is a membrane amino acid transporter that mediates the sodium-dependent antiport of neutral amino acids such as glutamine. ASCT2 facilitates glutamine uptake in tumor stem cells and promotes tumor growth in pancreatic ductal adenocarcinoma (PDAC) [97]. Increased expression of ASCT2 has been observed in several tumor types, including colorectal cancer, non-small cell lung cancer, pancreatic ductal carcinoma, and also breast cancer, and is associated with a poor prognosis. Based on this evidence, it can be concluded that ASCT2 plays an important role in tumorigenesis and is an attractive candidate for antitumor therapy [90]. The glutamine transporter SNAT2 is upregulated in hypoxia [98] and promotes glutamine uptake in cancer cells. Interestingly, SNAT2 compensates for ASCT2 loss of function [99]. In addition, ASCT2 and LAT1 function as coupled transporters, in which influx through one transporter is coupled to efflux through the other transporter [100].

Expression of CAIX in NSCLC

CAIX expression in lung carcinoma (Figures 3A–3C) is correlated with a high stage of tumor, necrosis, and low therapy efficiency [101, 102]. Multiple articles have described that, in comparison to healthy lung tissue, increased levels of CAIX mRNA and protein are often detected in resected NSCLC, even in early-stage tumors. The expression of CAIX is particularly frequent in squamocellular lung carcinoma. Several studies, detailed below, link a high CAIX level in NSCLC tumors to a worse prognosis.

In the study by Simi et al. (2006), *CA9* mRNA expression was confirmed in the vast majority of NSCLC samples [103]. Its levels significantly correlated with mRNA levels of VEGF and MMP9. High expression of CAIX predicted a worse prognosis for patients with squamocellular lung carcinoma as well as for patients with adenosquamocellular lung carcinoma and those with advanced NSCLC (G2–G3, T2–T3, Stage 2–3, and pN+ patients) [103]. CAIX expression was confirmed in 72% of early-stage NSCLC tumors (stage I and II) and was linked with higher cell proliferation assessed by the Ki-67 index. The extent of CAIX was significantly linked with disease recurrence and poor disease-free survival [104]. Using TMA sections, CAIX was detected in 62% of NSCLC tumor samples [105], and its level increased with higher tumor grade. CAIX as well as HIF1α expression was higher in SCC than in adenocarcinoma. Andersen et al. (2011) also detected a high CAIX expression in the SCC subgroup of NSCLC with a tendency toward negative prognosis [106]. Authors performed extensive tissue microarray analysis of tumor biopsies containing neoplastic epithelial cells and biopsies of tumor stroma comparing prognostic impacts of HIF1α, HIF2α and HIF targets, GLUT1, LDH5, and CAIX according to their tumor cell and stromal cell localization. HIF1α expression in tumor cells was an independent predictor of poor prognosis and, in contrast, a high stromal HIF1α expression indicated a good prognosis. These results show the impact of the TME and crosstalk among its components [106].

Hypoxic microenvironment containing CAIX-positive cancer-associated fibroblasts (CAF) was investigated in lung adenocarcinoma [107]. 26% of clinical samples had CAIX-positive CAFs, and patients with CAIX expression in CAFs had a significantly shorter period before recurrence than patients with CAIX-negative CAFs. Based on the immunohistochemical analysis, samples with CAIX-positive CAFs

had a higher number of tumors-promoting stromal cells, CD204⁺ tumor-associated macrophages and podoplanin-positive CAFs, and higher expression of Glut1 in cancer cells. The findings emphasize the importance of stromal components of the environment in promoting aggressive behavior.

After analysis of 98 tissue samples of resected primary NSCLC tumors, a strong association between CAIX and FOXP3+ regulatory T cell (Treg) abundance within the tumor was observed [108]. Treg cells regulate cytotoxic activity of T cells and their number increases in cancer patients. High Treg presence within tumors is linked with poor prognosis and infiltration of tumor stroma of NSCLC by FOXP3⁺Tregs is an early event linked with NSCLC progression. A relation between CAIX and PD-L1 was not detected [108]. Patients with high CAIX level and low tumor-infiltrating lymphocyte score had significantly worse survival. According to Giatromanolaki et al. (2020), CAIX is involved in immunosuppression pathways and targeting CAIX should be investigated in order to improve the immunotherapy efficacy [108].

CAIX expression plays an important role in NSCLC and is induced in hypoxic-necrotic areas. CAIX expression is linked to the expression of proteins and factors involved in angiogenesis, growth induction, and cell-cell adhesion disruption (EGFR, c-erbB-2, MUC1, p53, VGFR), which explains a strong association of CAIX with poor survival of patients at the early stage of NSCLC. CAIX expression was revealed as a significant factor of worse prognosis independent of angiogenesis indicating that even a weakly angiogenic tumor growth can be lethal for various reasons [109]. Failure of angiogenesis mediated by VEGF, e.g. due to the presence of endogenous angiogenesis inhibitors, such as angiostatin, due to the irregular perfusion of newly formed vasculature or oxygen consumption by fast-growing tumors, can be responsible for the focal origin of stricter hypoxia and for the induction of CAIX and angiogenic factors, such as PD-ECGF [110]. Longer median survival was reported for patients with NSCLC tumors positive for angiostatin compared to patients with angiostatin-negative tumors (146 weeks vs. 77 weeks).

CAIX can serve as a useful marker for distinguishing preneoplastic bronchial lesions and NSCLC. All morphologically normal epithelium of lung tissue, except for some in the near vicinity of tumors, as well as preneoplastic bronchial lesions (hyperplasia of basal cells, metaplasia, and dysplasia) exhibited a negative expression of CAIX. In contrast, carcinoma *in situ* and microinvasive carcinoma were CAIX positive [111].

Stewart et al. (2014) evaluated factors associated with the duration of the relapse period at NSCLC of stage I–II (N0 vs. N1). Nodal stage and membrane CAIX (mCAIX) immunohistochemical staining were the strongest independent predictors of a shorter relapse-free period at resectable NSCLC. mCAIX correlated with tumor size, proliferation markers, tumor necrosis, and genetic characteristics of a tumor. The presence of CAIX could help to select patients with a high risk of relapse that would benefit from adjuvant therapy. The tumor subtype can also be important. A very low CAIX expression was detected in lung adenocarcinoma with a high papillary component [112]. High CAIX expression in tissue was also a factor of poor prognosis in patients with resectable NSCLC in the study of Ilie et al. (2010) [113]. The highest expression of CAIX was detected in squamocellular lung carcinoma and large-cell lung carcinoma and the

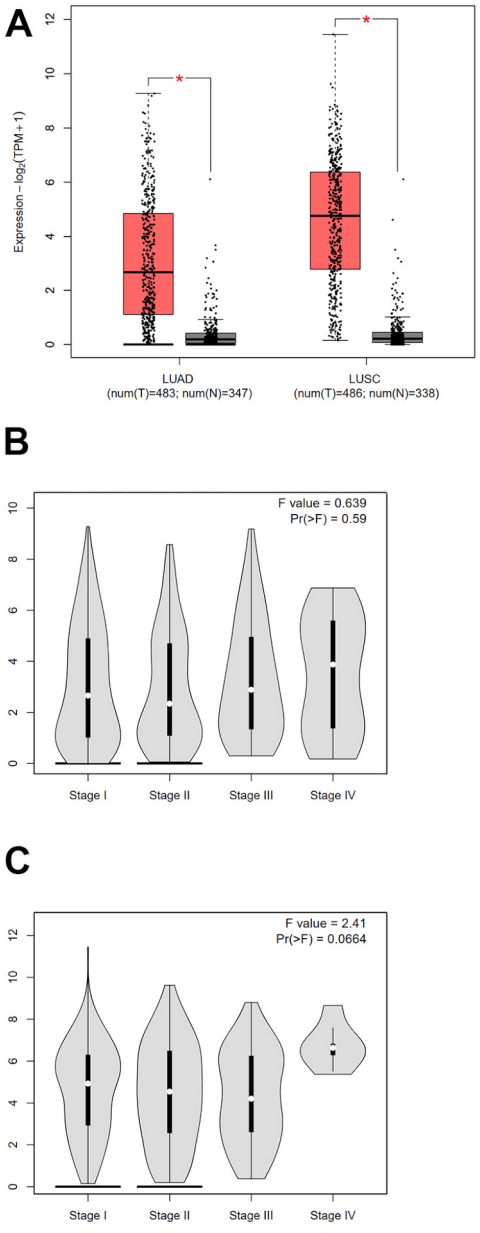

Figure 3. The analysis of CA9 mRNA level in lung carcinoma and normal lung tissue performed in GEPIA2-Gene Expression Profiling Interactive Analysis tool. A) In comparison with normal tissue samples, the CA9 level is increased in lung adenocarcinoma (LUAD) as well as in lung squamous cell carcinoma (LUSC). B) Comparison of CA9 expression in various stages of LUAD. C) Comparison of CA9 expression in various stages of LUSC.

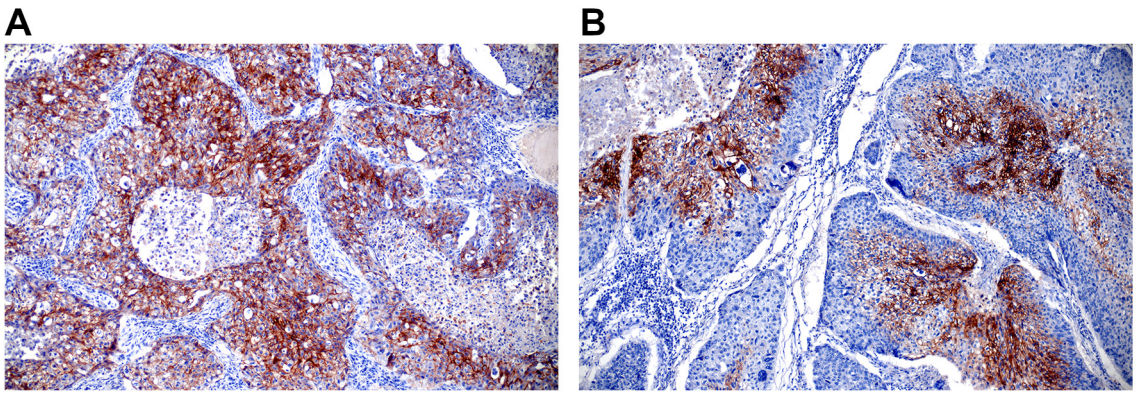

Figure 4. Representative immunohistochemical staining of carbonic anhydrase IX (CAIX) expression in non-small cell lung cancer. A) tissue section of adenocarcinoma B) tissue section of squamous cell carcinoma. Tissue slices were immunohistochemically stained with M75 monoclonal antibody using Dako EnVision®FLEX+detection system and counterstained for hematoxylin.

lowest was in lung adenocarcinoma (Figures 4A, 4B). This finding is probably linked with the necrosis, which often arises in squamocellular carcinoma and CAIX is frequently expressed around necrotic areas. This perinecrotic expression reflects the connection between CAIX in tumor tissue and hypoxia [114]. Endogenous tumor hypoxia markers polo1, CAIX, VGFR, and MMP9 were studied as prognostic factors at resectable NSCLC at stage I and II. HIF1α and CAIX were highly expressed in the regions with medium and high tumor necrosis in comparison with the regions with minimum necrosis, which confirms their relation to hypoxia. Expression of HIF1α and CAIX was significantly associated with a shorter disease-free survival. CAIX expression was the most reliable hypoxia marker to predict aggressive tumor behavior [115].

Patients in the early stage of NSCLC undergo operation. Patients in the advanced stage (stage III) with metastasis in mediastinal lymphatic nodes are treated by multinodal therapy (chemotherapy, radiotherapy, surgery). Disease progression during chemotherapy or disease relapse after surgery is most frequently caused by chemoresistance induced by several mechanisms. Chemotherapy induces intracellular acidosis and CAIX protects cells from apoptosis by maintaining normal intracellular pH in response to chemotherapy. Extracellular acidosis linked to CAIX activity is responsible for the chemoresistance of cancer cells. The majority of anti-cancer drugs, including vinorelbine, are charged weak bases and they become protonated and destroyed in acidic extracellular pH. The inhibition of HIF1 and CAIX can improve the prognosis of patients in the IIIA stage of NSCLC after chemoradiotherapy [116].

Tumor cells are characterized by increased glucose consumption and glycolysis. Increased glucose consumption leads to tumor acidification, which presents an obstacle for therapy. A relation between CAIX expression and fluorodeoxyglucose (FDG) intake detected by positron emission tomography (PET) was analyzed. The results showed a significant correlation between FDG intake and CAIX expression, which provides indirect proof for the co-transcription of glucose transporter and hexokinases, that control glycolysis, and CAIX induced by HIF1. This indicates that it could be possible to identify NSCLC patients by FDG-PET that could benefit the most from CAIX-targeted therapy [117].

In order to identify cell surface markers of lung cancer ten genes, including *CA9*, were selected based on differences in their mRNA expressions between lung tumor and normal lung tissue [118]. Protein expression was confirmed by immunohistochemistry. High levels of five out of ten markers, namely *CA9, CA12, CXorf61, LYPD3,* and *SLC7A11*, were significantly associated with worse survival when data were dichotomized. In their study, Skrzypski et al. *(*2013) stated that various histopathological types of NSCLC have different molecular features as the expression of half of the analyzed genes differed between NSCLC subtypes (SSC and adenocarcinoma). Expression of CSF1, DUSP6, MMD, and STAT1 differed between NSCLC and normal lung. Authors also confirmed an increase in *CA9* mRNA in SCC and AC compared with normal lung tissue, but in contrast to other studies, their results indicated that low *CA9* level was associated with poor prognosis in both SCC and AC, albeit this finding was not statistically significant [119]. Search for new prognostic factors of NSCLC to complement currently used clinicopathological data showed a relevance of the combination of the expression of Polo-Like Kinase 1, which is a main regulator of mitotic cell division and is involved in DNA damage response, TP53 mutation status, and CAIX protein level [120]. Both Plk1 and CAIX were upregulated in NSCLC adenocarcinoma samples compared to normal lung tissue. Patients with high CAIX levels had significantly shorter survival than patients with negative or low CAIX tumor expression. The combination of Plk1 and CAIX levels, and mutation in TP53 appears to be a promising predictive biomarker panel significantly influencing the overall survival in untreated as well as treated patient group (neo-adjuvant

and/or adjuvant therapy, including chemotherapy and/or radiotherapy). This finding indicates that patients with aberrant expression/mutation of the three markers would probably not benefit from standard (neo)adjuvant therapy.

There is little information available about the amount of the extracellular domain of CAIX shed into the blood of NSCLC patients. Hypoxia-caused chemoresistance and radioresistance worsen disease prognosis of NSCLC patients [121]. Pre-therapy plasmatic concentrations of osteopontin (OPN), VEGF, and CAIX before radiotherapy or chemotherapy administration were measured. High plasmatic levels of OPN, VEGF, and CAIX were significantly associated with a shorter overall survival. The combination of two markers additively doubled the risk of death, and high plasmatic concentrations of all three markers OPN/VEGF/CAIX led to a 5.9-fold increase in the risk of death. Multivariate analysis showed that the combination of the three markers OPN/VEGF/CAIX is an independent prognostic factor besides stage T or node status N [121]. The level of soluble CAIX (sCAIX) was evaluated in a cohort of over 200 NSCLC patients [114]. The level of sCAIX of cancer patients in plasma was significantly higher than in healthy individuals. A high level of sCAIX in plasma was associated with shorter OS and DSS and was an independent prognostic biomarker, particularly in the early stage of NSCLC (I and II). Plasma level of sCAIX significantly correlated with tumor size. There was no association with age, sex, smoking, histological type, or subtype. In contrast, Han et al. (2013) did not detect any difference between plasma levels of CAIX in NSCLC patient samples and control samples [122].

As a transmembrane protein, CAIX is easily accessible by antibodies or small molecule inhibitors. The drug carriers most frequently used for targeted therapy are liposomes, which provide crucial advantages such as good biocompatibility, low toxicity, and immunogenicity [123]. Docetaxel (DTX) is an anti-tumor drug commonly used for NSCLC treatment. The formation of immunoliposomes by connecting antibodies with the liposomal surface opened a new way for targeting tumor cells [124]. Immunoliposomal docetaxel targeting CAIX showed the strongest inhibitory effect on the growth of CAIX-positive lung carcinoma cells compared with non-targeted liposomal docetaxel or free docetaxel solutions. Immunoliposomal drugs can be administered via the intratracheal way for direct administration into lung tumors [125]. Triptolide-loaded liposomes conjugated with anti-CAIX antibody showed an improved uptake by CAIX expressing NSCLC A549 cancer cells and spheroids and an increased cell-killing effect when compared with free triptolide or liposomes without antibody [126]. Triptolide is a cytotoxic drug isolated from the Chinese herb *Tripterygium wilfordii*. In *in vivo* orthotopic lung tumor model in mice, CAIX targeting liposomes delivered via the pulmonary route concentrated in the lung, remained there even at 96 h, and exhibited improved efficiency in suppressing tumor growth and prolonging the survival of mice.

Expression of CAIX in fluidothorax

Pleural effusion can be encountered not only in diseases of the pleura and lungs but also in other diseases. It occurs when a pathological amount of fluid accumulates in the pleural cavity as a result of exceeding the resorption capacity of the pleura by more than 700 ml/day or in the case of pathologically reduced resorption.

The most common cause of pleural exudate is lung cancer, breast cancer, and pleural metastases. Also, infections in the pleural space during pneumonia, tuberculosis, and empyema are frequent causes. Pulmonary embolization, acute pancreatitis, and systemic diseases, most often rheumatoid arthritis and systemic lupus, are accompanied by pleural effusion with the character of an exudate [127].

Pleural effusions have a multi-factor etiology, and no cause is identified at approximately 5–10% of effusions. Malignant pleural effusions in cancer patients indicate locally advanced or metastatic disease. Biochemical analysis of fluidothorax is done to distinguish transudate, which occurs as a result of a violation of systemic factors, from exudate. However, existing biochemical markers have a high sensitivity, but low specificity, therefore, the diagnostic efficiency is not reached.

Cytological examination of fluidothorax is a common examination method but its sensitivity is about 50–60%. Low sensitivity can be a consequence of a low portion of malignant cells in fluidothorax and problems in distinguishing between benign cancer cells and atypical reactive mesothelial cells. Traditional tumor markers, including carcinoembryonal antigen (CEA), cancer antigen (CA15-3, CA19-9, and CA125), and cytokeratin fragment (CYFRA 21-1), are limited to specific types of tumors. From these markers, the best results are obtained by the examination of CEA in pleural effusions. CEA sensitivity at diagnostics of malignant pleural effusion is 35–63.6% [128].

CAIX expression allows tumor cells to adapt to acidosis and supports disease progression. Therefore, CAIX determination in fluidothorax could be a suitable diagnostic marker to distinguish between benign and malignant effusions. Liao and Lee (2012) monitored the CAIX expression in fluidothorax of different etiology by ELISA and immunocytochemistry. They found out that malignant pleural effusions are characterized by increased levels of CAIX. The value of 1882 pg/dl served the best for deciding between benign and malignant effusions. No benign effusion showed CAIX positivity. All effusions exhibited a significant positive correlation between the CAIX concentration measured by ELISA and the CAIX expression determined by ICC [129]. Tuberculous pleuritis is a frequent cause of false positive results of tumor markers, including Cyfra 21-1, CEA. In Liao and Lee's study (2012), levels of CAIX in three tuberculosis samples were <2000 pg/dl and ICC showed CAIX negativity. The use of ELISA for CAIX level measurements can decrease errors during sample collection and the combination of two methods can provide higher diagnostics efficiency [130]. In

another study by Liao et al. (2011), 150 pleural effusions were immunocytochemically stained for CAIX and GLUT1. The majority of examined malignant effusions were connected with lung cancer (32 of 47) as well as 14 out of 38 probable malignant effusions were of lung cancer etiology. All benign effusions were negative for both proteins but malignant effusions and probable malignant effusions showed CAIX positivity in 63.8%, and GLUT1 was positive in 74.5%. A significant positive correlation between CAIX and GLUT1 was detected. When both markers were combined the diagnostic performance was higher (sensitivity 76.6%, specificity 100%), suggesting that immunocytochemical staining of CAIX and GLUT1 could be a helpful tool in identifying malignant cells in pleural effusions [130].

In another study, the authors monitored the expression of CAIX in 71 pleural effusions including 59 malignant effusions from various cancer patients and from 12 patients with benign disease by RT-PCR method to detect the *CA9* gene expression. *CA9* positivity was found in 53 out of 59 samples (89.8%) of pleural effusions of all cancer patients and in 10 out of 11 lung cancer patient effusions. The sensitivity and specificity of *CA9* gene expression was 89.8% and 91.7%, respectively [131].

Tumor microenvironment and models used in preclinical research

For many decades, two-dimensional (2D) culture models, i.e. cells growing in a monolayer, have been used as a tool for analyzing molecular signaling and for evaluating the biological efficacy of investigated bioactive molecules *in vitro*. Growth in 2D monolayers gives cells access to a similar amount of nutrients and growth factors present in the culture medium, resulting in homogeneous cell growth and proliferation. This feature makes 2D models attractive mainly due to their simplicity and efficiency [132]. However, studying the TME *in vitro* in 2D cell cultures does not sufficiently reflect the mutual relationships within the TME [133]. The biggest shortcoming of 2D cultures is the absence of stroma, which is important when modeling tumor tissue, and they also do not sufficiently reflect other conditions of the TME, such as cellular heterogeneity, gradient of nutrients, oxygen and pH, interactions between cells, or matrix composition [134].

Three-dimensional cellular models-spheroids. An intermediate step between *in vitro* and *in vivo* models is represented by spheroids. The main advantage of 3D cultures is their defined spatial geometry. 3D cultures are able to better mimic not only *in vivo* morphology but also cellular connections, polarity, gene expression, and tissue architecture. Moreover, 3D cultures are composed of cells with different phenotypes, such as proliferating, non-proliferating, and necrotic cells. They are better able to mimic spatial architecture as well as physiological responses [135, 136].

Spheroids closely mimic the *in vivo* features of solid tumors. Beyond a critical size (about 500 μm) spheroids represent characteristic features of avascular tumors with an external proliferating zone, an internal quiescent zone, and a necrotic core due to gradients of nutrient and oxygen concentration [137].

The arrangement of heterogeneous cell populations in spheroids and the establishment of pathophysiological gradients are also comparable to the situation in micrometastases, microregions of poorly vascularized tumors, or intercapillary tumor regions [138]. The spherically symmetric geometry of spheroids allows a direct comparison of structure with function, as microenvironmental gradients that determine spheroid morphology are spatially correlated with changes in cellular physiology, which has several advantages for the use of spheroids as *in vitro* tumor models. Such symmetry of spheroids allows prediction of radiation response, drug penetration, binding/activity, and it can also aid in the interpretation of regulation of cell physiology, proliferation, and viability [139].

Another approach is the use of animal models. Apart from the ethical aspect of the use of animals in experimental practice, the fact that in animal models human tumors are embedded in a microenvironment composed of animal cells must be taken into account [140]. This chimeric environment is unlikely to represent the physiology of a human tumor tissue. The failure rate in the translation of anticancer drugs from animal testing to human treatments remains over 97% [141]. Despite the fact that the reasons for terminating clinical trials are various, the high failure rate illustrates the need for better models for the pre-clinical characterization of potential anticancer drugs.

Single- and multi-component spheroids. Our experience with the differences between 2D and 3D models led us to prepare 3D models that incorporate multiple TME components. The goal of our research is the CAIX protein. The expression of CAIX is mainly driven by a decrease in the level of oxygen, and in the TME this protein plays a very important role in the homeostasis of the intracellular pH, which must be maintained in the physiological range of pH 7.2–7.4. When simulating hypoxia in a hypoxic incubator, all cells are exposed to the same concentration of oxygen, unlike in tumor tissue, where an oxygen gradient arises depending on the distance from the nearest blood vessel. As a result of hypoxia, a gradient of pH, nutrients, and waste products also arises in the 3D structure of the tumor tissue. Lung adenocarcinoma cell line A549 expresses CAIX protein in hypoxic conditions [114]. As CAIX is localized extracellularly and its expression is associated with hypoxic conditions in tumor tissue, CAIX also represents a potential therapeutic target [30]. Humanized antibodies specific to the CAIX protein [142] are currently being tested in the preclinical phase. These antibodies are able to inhibit the enzymatic function of CAIX, but at the same time, they are also able to induce antibody-dependent cell cytotoxicity (ADCC). A model incorporating multiple components of the TME is indispensable for testing the effectiveness of new therapeutics. It is clear that it is not

possible to prepare an ideal *in vitro* model replicating the heterogeneity of tumor tissue but we can prepare a model with several tumor and non-tumor TME components as well as pH and oxygen gradients. 3D model of spheroids has been shown to have some features relevant to patient NSCLC tumors. The properties of the spheroids strongly depend on the cell composition. The simplest 3D model is represented by multicellular spheroids generated from one NSCLC cell line. We detected a high level of CAIX protein (Figure 5A) in single-component 3D models of spheroids (see Supplementary file) formed only by A549 tumor epithelial cells. CAIX expression was predominantly in the hypoxic central regions of the spheroid. Such a model does not include non-tumor components of the TME, which, as was shown above, represent an integral part of the TME and significantly influence not only tumorigenesis but also the response to antitumor drugs in NSCLC. Therefore, the 3D model with the incorporation of lung fibroblasts is another step towards enriching the TME spheroid with stromal components. Such an approach represents a higher level of *in vitro* 3D models. Complex heterotypic spheroid models contain two or more cell types. Moreover, MRC5 fibroblasts are derived from lung tissue [143]. In such a model, it is possible to analyze different ratios of the tumor and stromal components, which change in NSCLC depending on the tumor stage as well as the mutation load [144]. A heterotypic 3D model composed of A549 NSCLC cell line and MRC5 fibroblasts is documented in Figure 5B. The presence of fibroblasts incorporated in the A549 spheroid is evidenced by alpha-smooth muscle actin; in the tumor cells, we detected CAIX protein (see Supplementary file). The immune component of the TME significantly influences the response to antitumor treatment and is also important when testing the effectiveness of new potential therapeutics. Homo- as well as heterotypic spheroids can be further co-cultured with immune cells isolated from peripheral blood. Such a model is suitable for testing the penetration of PBMC into the spheroid, for the analysis of the polarization of TAMs, immune checkpoint proteins in the spheroid,

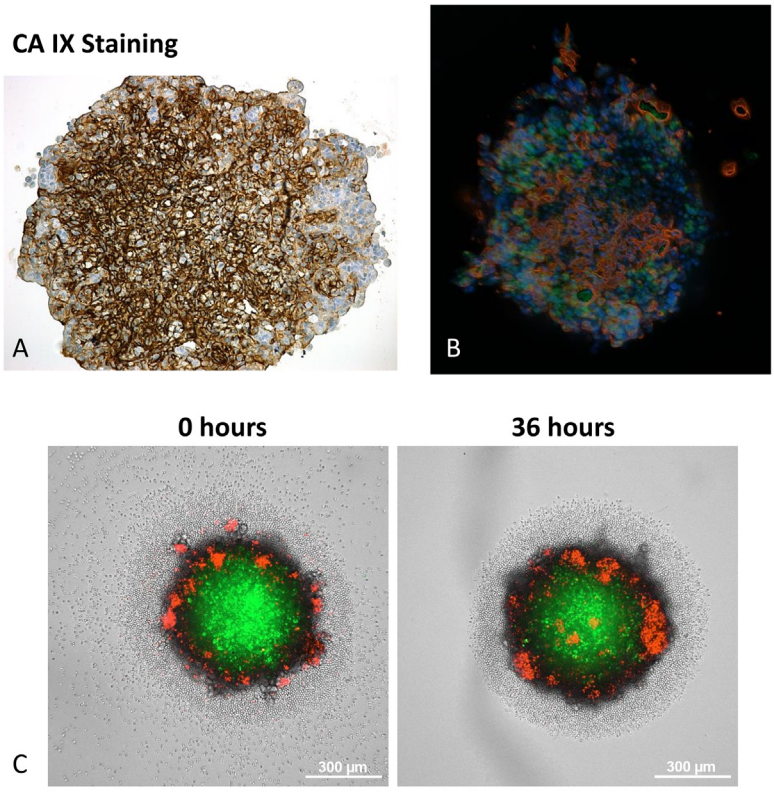

Figure 5. 3D models of the tumor adenocarcinoma cell line A549. A) Representative immunohistochemistry image of carbonic anhydrase IX (CAIX) expression in single-component A549 spheroids. Spheroids were harvested on day 8, fixed with 4% PFA, paraplast-embedded, and immunohistochemically stained with M75 monoclonal antibody using Dako EnVision®FLEX+detection system and counterstained for hematoxylin. B) Spheroids generated by co-culturing lung tumor cells A549 and lung fibroblasts MRC5. Spheroids were harvested on day 8 and labeled using anti-αSMA primary antibody MCA5781GA (Bio-Rad) and Alexa Fluor™488 secondary antibody (green), anti-CAIX conjugated M75-AF555 antibody (red), nuclei were labeled with Hoechst (blue) and detected by immunofluorescence using BioTek Cytation C10. C) Multicomponent spheroids: penetration of peripheral blood mononuclear cells (PBMC) into A549/MRC5 spheroids. PBMC were labeled with CellBrite™ Orange (red), lung tumor cells A549 and lung fibroblasts MRC5 were labeled with CellBrite™ Green (green), and penetration of PBMC was detected by immunofluorescence using BioTek Cytation C10 for a period of 36 h.

as well as for the evaluation of the response to therapeutics. NSCLC is characterized by an immunosuppressive microenvironment formed due to multiple mechanisms – secretion of anti-inflammatory cytokines and mediators, activation of inhibitory immune checkpoint mechanisms [145], and polarization of M2 TAMs. In the 3D model of heterotypic spheroids, it has been shown that co-culture of lung cancer cells and lung-derived CAFs promoted the M2 polarization of THP-1 monocytes [146]. Figure 5C shows the penetration of immune cells (PBMC) into heterotypic spheroids formed from A549 NSCLC and MRC5 fibroblasts (see Supplementary file).

NSCLC spheroids, homo- or heterotypic, are used for the evaluation of novel drug candidates, for studies of signaling pathways in cancer cells, and in the analysis of interactions between cancer cells and other cell types in the TME.

Currently, the center of interest is the preparation of 3D models of tumor organoids created directly from the patient's tumor tissue. It is the next step in the personalized, precise treatment of tumors. Patient-derived organoids (PDO) retain the mutational burden and characteristic features of tumor tissue. There are several studies demonstrating the feasibility of patient-derived organoids for anticancer drug screening. These NSCLC organoids express adenocarcinoma markers and demonstrate a similarity of response to cisplatin with spheroids composed of NSCLC H1299 cells [147, 148].

The NSCLC spheroid model shows several physiological and molecular characteristics of tumor tissue and represents a suitable model for testing not only potential new drugs but also signaling pathways, interactions, and changes in the TME. In addition to well-defined and characterized NSCLC tumor cell lines, the tumor tissue itself is also a suitable source of cancer cells. The PDO best represents the heterogeneity of the tumor tissue. However, a correlational analysis is needed between the therapeutic outcome and the testing of drugs for PDO, when the type of NSCLC, mutation load, and cancer stage need to be taken into consideration.

In conclusion, the TME is very heterogeneous and characterized by dynamic interactions between its individual components. It plays a fundamental role in all processes of tumorigenesis, through the expression of specific markers, genetic changes, and even the response to anticancer therapy. Therefore, knowledge of its individual parts, their interconnections and interactions represent a very important step not only toward a more precise diagnosis, but also toward targeted and tailored personalized therapy. CAIX protein plays a crucial role in the process of adaptation of tumor cells to the changes in important physical and chemical parameters (low oxygen concentration, low pH) in NSCLC as well. As described above, the results of a number of studies indicate that the CAIX protein could represent a potential diagnostic biomarker in NSCLC tumor tissue and pleural effusion and, at the same time, due to its membrane localization and high expression preferentially in hypoxic tumor tissue, also an effective therapeutic target.

Supplementary information is available in the online version of the paper.

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Can hypoxia marker carbonic anhydrase IX serve as a potential new diagnostic marker and therapeutic target of non-small cell lung cancer?

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Supplementary Information

Isolation of PBMCs and culture of spheroids in the presence of PBMCs

Peripheral blood with anticoagulant was mixed with DPBS (Biotech) in a ratio of 1:1. The diluted blood was applied to the surface of Ficoll, while the ratio of Ficoll to blood was 2.4:3, and centrifuged at 400×g for 35 min. After centrifugation, we created a gradient, where the upper layer was plasma, the lower layer was represented by Ficoll, and in the middle was a white layer with PBMC (Peripheral Blood Mononuclear Cell). The PBMC layer was carefully removed, without plasma and Ficoll. The PBMC suspension was washed 3× with DPBS and centrifuged at 180×g for 10 min, the supernatant was removed, the sediment was resuspended in DMEM, and the cells were counted. Subsequently, the isolated PBMCs were cultured with heterotypic A549/MRC5 spheroids in a ratio of 1 (PBMC) to 2.5 (spheroid cells). Spheroids were cultured with immune cells for 5 d in DMEM and were subsequently analyzed by Cytation C10 (Agilent Technologies).

Homotypic spheroids

Homotypic spheroids were prepared from the A549 cell line (A549-CCL-185-ATCC). At the beginning of the experiment, the cells were counted and a cell suspension with a concentration of 100 cells/µl was prepared. 10 ml of PBS was added into the dishes for the culture of suspension cells and drops with a volume of 20 µl of the prepared cell suspension were pipetted onto the lid of these plates using an 8-channel pipette. Droplets prepared in this way were cultured in a cell incubator for 96 hours at 37 °C, 5% CO_2. Subsequently, the spheroids formed in the drops were transferred to a dish with a non-adherent surface and DMEM culture medium supplemented with 10% FCS (HyClone Laboratories) and gentamicin (Sandoz) and cultivated for 5 more days in a cell incubator.

Heterotypic spheroids

We prepared co-culture spheroids by culturing NSCLC A549 with MRC5 lung fibroblast cells (MRC5-CCL-171-ATCC). Epithelial tumor cells and MRC5 fibroblasts were mixed in a 1:1 ratio. A suspension of cells in DMEM with a concentration of 20 cells/1 µl was applied to individual wells of a 96-well plate in a volume of 100 µl/well. Before mixing, the cells were labeled with a green dye (CellBrite Green, Biotium). The cell suspensions prepared in this way in ULA (Ultra Low Attachment) plates were cultured for 4 d. After the formation of co-culture spheroids, they were co-cultured with PBMCs for 3 d. The penetration of PBMCs into spheroids was analyzed using a Cytation C10 confocal reader (Agilent Technologies).

Fixation and embedding of spheroids

Spheroids were fixed with 4% paraformaldehyde (Sigma Aldrich) for 1 h at room temperature. Before and after fixation, the spheroids were washed with PBS. Washing was followed by sequential dehydration with increasing series of alcohols: 30% ethanol for 3 min, 50% ethanol for 15 min, 70% ethanol for 5 min, eosin in ethanol for 1 min, 70% ethanol for 2×5 min, 80% ethanol for 3 min, and 96% ethanol (CentralChem) for 15 min. This was followed by washing in isopropanol for 10 min and transferring the spheroids to metal trays. After isopropanol aspiration, xylene (CentralChem) was added to the spheroids for approximately 3 min. The spheroids in the metal trays were then transferred to an embedding station (Leica) where they were saturated in paraffin I and II at 56 °C for 20 min. Finally, the spheroids were embedded in paraffin and allowed to solidify. From formed paraffin blocks, 4 µm sections of spheroids were prepared and placed on slides, which were further used for immunohistochemical analysis.

Immunohistochemical staining

CAIX protein was immunohistochemically labelled on A549 spheroid sections using the Dako EnVison FLEX+ System, HRP kit. Slide sections were first deparaffinized with: xylene 2×5 min, 96% ethanol 2×5 min, 70% ethanol 5 min, and dH_2O 5 min. After blocking endogenous peroxidase activity for 5 min using Peroxidase Block solution (Dako) and washing in Washing Buffer (EnVision™ Flex, Dako) for 5 min, sections were incubated for 1 h with primary antibody M75 (hybridoma medium) [Pastorekova et al., 1992] diluted 1:100 in Antibody Diluent (Dako). After washing for 5 min, the spheroid section samples were incubated with anti-mouse-HRP secondary antibody (Dako) for 30 min and washed again with wash buffer for 1×3 min and 1×10 min. Labelling was visualized using 3,3´-diaminobenzidine (DAB) as chromogenic substrate by a minute incubation, after which the samples were washed for 5 min in dH_2O to stop the reaction. We used hematoxylin (Dako) to label the nuclei for 6 min and then washed them under running water. The slides with sections of spheroids were mounted in Aquatex (Merck) medium. The prepared slides were further analyzed using a Leica DM4500B microscope with a Leica DFC480 camera.

Immunofluorescence

Live spheroids were labeled in phenol red-free medium. Spheroids were first labeled with Actin alpha smooth muscle antibody (Bio-Rad MCA5781GA) diluted 1:300 for 1 h at 37 °C, then the spheroids were washed and labeled with Alexa Fluor™ 488 secondary anti-mouse antibody (Invitrogen A21202) diluted 1:1000 for 1 h at 37 °C. After washing, spheroids were labeled with monoclonal antibody M75 conjugated with AlexaFluor555 diluted 1:100 for 1 h at 37 °C. After 50 min, we added Hoechst 33342 (Invitrogen H3570) diluted 1:1000 for 10 min at 37 °C. Finally, the spheroids were washed and observed using CytationC10 (BioTek).

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