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Review

The roles of carbonic anhydrases IX and XII in cancer cell adhesion, migration, invasion and metastasis

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The main function of carbonic anhydrases (CAs) in cancer cells is the pH regulation through a conversion of H_2O and CO_2 to H^+ and HCO_3^-. However, the data of *in vitro* and *in vivo* studies have demonstrated that transmembrane isoforms of CA IX and CA XII are involved in various steps of cancer cell migration, invasion and metastasis. According to literature, inhibition of these CAs can affect the expression of multiple proteins. Some scientific groups have reported the possible interactions between CA IX and E-cadherin–catenin system, CA IX and integrins, CA IX, CA XII and ion transporters, which all are highly involved in cell-to-cell adhesion, the formation of membrane protrusions and focal adhesions. Nevertheless, CA IX and CA XII have a high impact on tumour growth and metastases formation. The data discussed in this review are quite recent. It highly support the role of CA IX and CA XII in various cancer metastasis processes through their interactions to other invasion proteins. Nevertheless, all findings show the great potential of these CAs in the context of research and application in clinical use.

Introduction

According to the World Health Organization, 8.8 million people died from cancer in 2015 and it was one of the leading causes of death worldwide [Siegel, Miller and Jemal, 2019]. In many cases, metastases are one of the main reasons for cancer treatment failure and the primary cause of cancer-related deaths [Zhao *et al.*, 2016]. Metastatic tumours are difficult to treat with conventional surgery or radiotherapy due to their anatomically diffuse localisation in different tissues and organs. In most cases, metastatic tumours are resistant to cytotoxic agents [Steeg, 2016]. The emergence of new cancer detection methods makes it possible for early diagnosis of solid tumours and allows for early treatment before they undergo metastasis. However, once cancers spread beyond the initial primary site, they are usually highly incurable and fatal [Guan, 2015].

Metastasis is a complicated biochemical process controlled by many signalling pathways and involves multiple sequential and interconnected steps. Metastasis proceeds in four steps: detachment, migration, invasion and adhesion [Guan, 2015]. Cancer cells first detach from the primary tumour by disassembling intercellular connections, then migrate through particular tissue by forming specific connections with the components of extracellular matrix (ECM), then invade through basal lamina to blood or lymphatic vessels and travel to different organs where they settle (adhesion step) and grow to secondary tumours [Han et al., 2012; Christine & Weinberg, 2013]. The formation of metastases is driven by various signalling pathways and is highly affected by the surroundings ECM and tumour microenvironment (TME). The growing number of evidence show that hypoxia might be the key factor in the formation of metastases [Brahimi-Horn, Chiche and Pouysségur, 2007; Lou *et al.*, 2011; Kopecka *et al.*, 2016].

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Abbreviations: CA, carbonic anhydrase; TME, tumour microenvironment; ECM, extracellular matrix; HIF-1α, hypoxia induced factor 1 alpha; pHi, intracellular pH; pHe, extracellular pH; ITGB1, integrin 1 beta; ITGA2, integrin 2 alpha; ROCK, RhoA-associated protein kinase 1; FA, focal adhesion; Hh pathway, Hedgehog pathway; SMO, smoothened protein; MMP, metalloproteinase; NHE1, sodium hydrogen transporter 1; NBCe1, sodium bicarbonate cotransporter 1; AE, anion exchanger

Tumour hypoxia is associated with poor prognosis in several cancer types [Eom *et al*., 2016]. Hypoxia-inducible factor 1α (HIF-1α) is one of the main elements that stabilise and alter the metabolism of tumour cells in hypoxia [Brahimi-Horn, Chiche and Pouysségur, 2007]. HIF-1α up-regulates a big variety of genes that are responsible for the protection of tumour cells against hypoxic stress [McDonald, Swayampakula and Dedhar, 2018]. One of the tumour biological responses to hypoxia is the induction of angiogenesis, resulting in the formation of a dysfunctional vasculature that serves to perpetuate poor perfusion and exacerbate hypoxia [Pavlova and Thompson, 2016]. The second important biological response to hypoxia is a dynamic adaptation of cancer cell metabolism. This enables the acquisition and use of nutrients and metabolites from an increasingly nutrient-poor and O_2-low environment, thereby maintaining viability and enabling continued proliferation [Pavlova and Thompson, 2016]. Active pH regulation is one of many hypoxic responses that allows tumour cells to maintain intracellular pH (pHi) homoeostasis, viability and proliferation in acidic TME. Cancer cells employ a multiprotein complex of enzymes and transporters to keep effective pH regulation. Critical components of this pH regulatory machinery are carbonic anhydrases (CAs), particularly the transmembrane isoforms: carbonic anhydrase IX (CA IX) and CA XII [Robertson, Potter and Harris, 2004; Parks and Pouysségur, 2017].

CA IX and CA XII are transmembrane proteins that catalyse the reversible dehydration of bicarbonate ($H_2O + CO_2 = H^+ + HCO_3^-$) [Brahimi-Horn, Chiche and Pouysségur, 2007; Tafreshi *et al*., 2016]. In recent studies, CA IX and CA XII were considered to be potent biomarkers of poor patient prognosis and treatment resistance for many types of solid tumours [Hsieh *et al*., 2010; Kaya *et al*., 2012; Parks and Pouysségur, 2017; Chen *et al*., 2018]. Several studies have demonstrated that both CA IX and CA XII are critical for the tumour cell migration and invasion [Svastova *et al*., 2012; Radvak *et al*., 2013; Swayampakula *et al*., 2017; Guerrini *et al*., 2018], and the growth of metastases [McIntyre *et al*., 2012; Tafreshi *et al*., 2012; Chen *et al*., 2018]. However, CA XII has not been investigated so thoroughly as CA IX, and there is not enough data on the participation of CA XII in some key steps of cancer cell migration, such as cell-to-cell and cell to ECM adhesion.

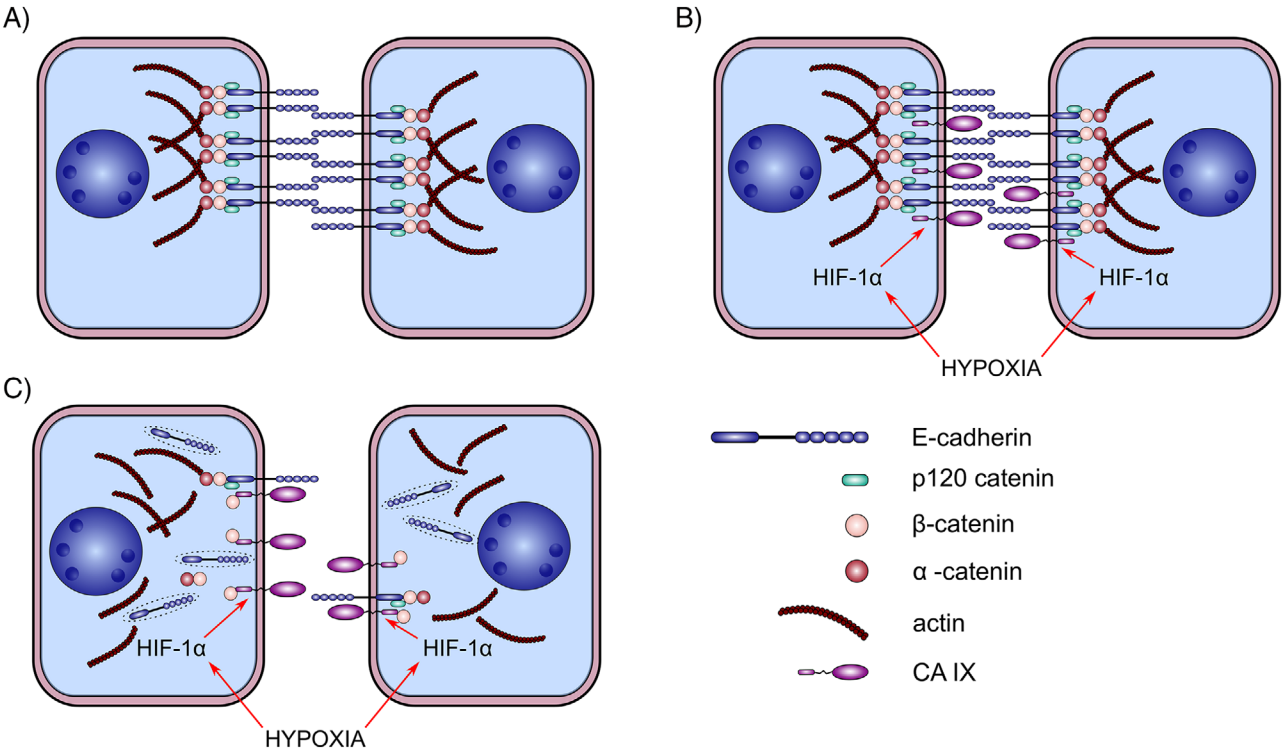
The role of CA IX in cancer cell–cell adhesion

In 1914, Theodor Boveri hypothesised that tumour cells go through important changes during cell adhesion while observing malignant tumour cells leaving the primary tumour and disseminating to distant organs [Cavallaro and Christofori, 2004]. This led to the opinion that cancer cell migration and invasion might be influenced by the changes in cell–cell and cell–matrix adhesion. Nowadays, it is already proven that changes in the interactions of cell-adhesion molecules can modulate signal transduction and contribute to the tumour progression by altering their adhesion properties [Wijnhoven, Dinjens and Pignatelli, 2000].

Epithelial tissues are composed of tightly bound cells, which attach to each other directly by cell–cell junctions. There are four major groups of cell–cell junctions – tight junctions, adherens junctions, desmosomes and gap junctions [Yap, Brieher and Gumbiner, 1997; Knust and Bossinger, 2002; Green *et al*., 2010]. Adherens junctions and desmosomes are also referred to as anchoring junctions, as they are anchoring sites for cytoskeletal filaments and link cytoskeletons of the adjacent cells [Green *et al*., 2010]. Adherens junctions are primarily mediated by cadherin proteins expressed at connection sites [Rankin and Giaccia, 2016]. Epithelial cadherin (E-cadherin) forms stable zipper-like junctions through homophilic intercellular binding [Jamora and Fuchs, 2002] (Figure 1A). The ectodomains of E-cadherin form dimers which are stabilised by extracellular calcium ions in the intercellular gap [Jamora and Fuchs, 2002]. The cytoplasmic domain of E-cadherin binds to actin cytoskeleton indirectly through an adaptor protein complex containing p120-catenin, β-catenin and α-catenin(Figure 1A) [Wijnhoven, Dinjens and Pignatelli, 2000; Žilka *et al*., 2003; Green *et al*., 2010; Mège and Ishiyama, 2017].

Mounting evidence has revealed that the down-regulation of E-cadherin decreases intercellular contacts and reduces cell polarity, and therefore promotes epithelial–mesenchymal transition [Song *et al*., 2019]. Reduced expression of E-cadherin is often

Figure 1 | Schematic view of possible interaction between CA IX and E-cadherin-mediated cell–cell adhesion
(**A**) Cell–cell connections mediated by cadherin at intercellular junctions. (**B**) Increased hypoxia induces HIF-1α, which induces CA IX expression. CA IX co-localises with E-cadherin in same areas of membrane at intercellular junctions. (**C**) CA IX accumulation induces the break of connections between E-cadherins by possibly interrupting the complex of E-cadherin and α-, β-catenins.



observed in metastatic tumours. In several experimental models, it has been demonstrated that down-regulation of E-cadherin can sufficiently promote metastasis of malignant cells [Evers *et al.*, 2006; Lu and Kang, 2010; Rankin and Giaccia, 2016]. In addition, either loss or mutation of E-cadherin or signal transduction-related destabilisation of E-cadherin–catenin interactions can result in reduced cell to cell adhesion, which is associated with increased tumorigenesis and cancer cell migration [Žilka *et al.*, 2003].

It is well known that CA IX and E-cadherin mainly localise in the plasma membrane (Figure 1B). Žilka et al. [2003] confirmed the co-localisation of CA IX and E-cadherin by double immunostaining and confocal microscopy in CA IX-transfected MDCK cells. Both proteins accumulated in the same areas of adherens junctions (Figure 1B). In the same study, the authors determined that CA IX-transfected MDCK cells had reduced adhesive properties and could not form cell aggregates compared with parental MDCK cells. Further, the authors proposed a possible direct interaction between CA IX and E-cadherin. Normally, in MDCK cells, E-cadherin binds to actin through α-catenin and β-catenin forming complexes (Figure 1A) insoluble by Triton-X, and therefore the free form of E-cadherin is not detected in cytoplasm. Whereas MDCK-CA IX cells showed decreased levels of insoluble E-cadherin as well as both α- and β-catenins after extraction by Triton-X. In addition, co-precipitation assay showed that in MDCK-CA IX cells, β-catenin co-precipitated with CA IX at higher levels than compared with E-cadherin. This suggested that β-catenin might competitively bind to CA IX instead of E-cadherin, and therefore decrease cell–cell adhesion and cell aggregation (Figure 1C) [Žilka *et al.*, 2003].

Kim et al. [2011] in their study demonstrated that CA IX has a negative effect on E-cadherin and α-catenin expression and distribution. In their study, human cervical cancer cells transfected with CA IX

(C33A/CA9) expressed lower levels of E-cadherin and α-catenin, compared with the parent C33A cells [Kim *et al*., 2011]. In addition, E-cadherin was diffusely distributed in low levels within the cytoplasm of C33A/CA9 cells, compared with the steady distribution in the cell membrane of C33A/Mock cells [Kim *et al*., 2011]. Later observation supports the possible interaction between CA IX and E-cadherin.

To conclude, scientific data show that increased expression of CA IX may disturb cancer cell-to-cell adhesion processes, through possible interaction with E-cadherin, but precise mechanism is not yet determined.

The role of CA IX and CA XII in cancer cell migration and invasion

A growing body of evidence supports the role of CA IX and CA XII as the key regulators of cancer cell migration, invasion and metastasis. Several studies have demonstrated that genetic depletion of CA IX and CA XII and pharmacological inhibition of CAs reduce the migration and invasion of various cancers cells [Hsieh *et al*., 2010; Svastova *et al*., 2012; Csaderova *et al*., 2013; Svastova and Pastorekova, 2013].

Available data demonstrate that either the knockdown of CA IX or pharmacological inhibition of CA IX in different breast cancer cell lines, under hypoxic conditions, can decrease the migration and invasion of the cells [Hsieh *et al*., 2010; Lock *et al*., 2013; Ward *et al*., 2015]. Though Chen et al. [2018] demonstrated that the knockdown of CA IX in triple-negative breast cancer cells (UFH-001) resulted only in decreased migration and not invasion. Other researches demonstrated that genetic or pharmacological inhibition of CA IX in human fibrosarcomas [Radvak *et al*., 2013], glioblastomas [Amiri *et al*., 2016] and ovarian cancer cells [Ward *et al*., 2015] resulted in decreased cell migration and invasion. These data confirm that the inhibition of CA IX, resulting in decreased cell migration and invasion, is not specific to breast cancer and the same results can be achieved with different cancer cell types.

Hsieh et al. [2010] in their research demonstrated that silencing CA XII gene also decreases breast cancer cell migration and invasion, though the effect was not so strong compared with the CA IX silencing. In addition, the same group determined that CA XII gene silencing decreased cell migration and invasion more significantly in MDA-MB-231 cell line, than compared with MCF-7 and Hs578T cell lines. This was possible because MDA-MB-231 cells express higher levels of CA XII compared with the other cell lines, thus making MDA-MB-231 cells more sensitive to CA XII silencing and inhibition. Guerrini et al. [2018] in their research demonstrated a possible indirect involvement of CA XII in cancer cell migration and invasion by inhibiting Smoothened protein (SMO) [Guerrini *et al*., 2018], which is the main signal transducer in Hedgehog (Hh) pathway. It is known that Hh pathway is responsible for tissue repair, homoeostasis and stem cell maintenance in healthy tissues, and can regulate cell migration, adaptation and survival in unfavourable microenvironment, such as increased hypoxia and decreased extracellular pH in tumour tissues [Scales and de Sauvage, 2009; Jia *et al*., 2018]. In this research, scientists observed that silencing or pharmacological inhibition of SMO in MDA-MB-231 cells, resulted in reduced CA XII expression, which possibly led to decreased migration of cancer cells [Guerrini *et al*., 2018].

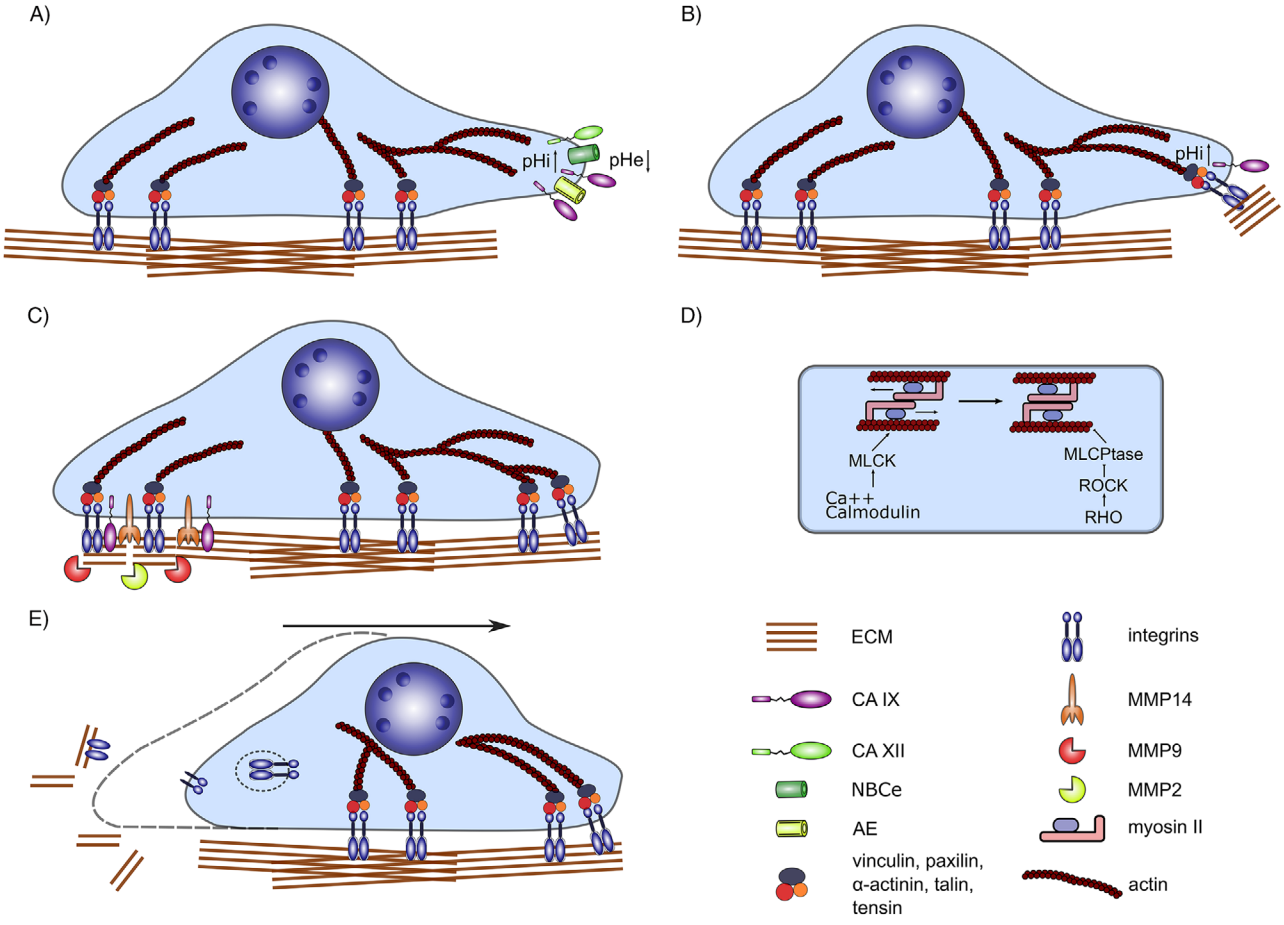
Some studies have shown that CA IX could be involved in the formation of migratory and invasive membrane protrusions and focal adhesions (FAs) during cancer cell migration through interactions with integrins [Swayampakula *et al*., 2017] and ion exchangers [Svastova *et al*., 2012; Svastova and Pastorekova, 2013]. In addition, it was reported that both CA IX and CA XII could interact with matrix metalloproteinases in the proteolysis of ECM during cancer cell migration and invasion [Hsieh *et al*., 2010; Swayampakula *et al*., 2017].

The role of CA IX in the FAs

According to scientific literature, most of the cancer cells migrate using mesenchymal migration model, which depends on the activity of proteases, stress fibres and integrins [Friedl and Wolf, 2003]. A mesenchymal cell migration defines a multi-step process of cell membrane protrusion formation, adhesion to the substrate, stabilisation at the leading edge, proteolysis of ECM structures, and lastly, cell body translocation and detachment of the cell's trailing edge (Figure 2) [Friedl and Wolf, 2003; Huttenlocher and Horwitz, 2011]. The whole migration process is mediated by a complex interactome of many proteins, and is reviewed in detail elsewhere [Friedl and Wolf,

Figure 2 | Schematic view of CAs involvement in individual cell migration through ECM

(**A**) Formation of protrusion (lamellipodia) of leading membrane edge at acidic pH level. CAs alongside with ion exchangers (not shown) establishes pH gradient. (**B**) Formation of a new focal contact via integrins. Integrin's cytoplasmic tail directly interacts with α-actinin, talin, the focal adhesion kinase, and other proteins, which let integrins to attach actin filaments inside the cell. (**C**) Degradation of trailing focal contact. At the trailing focal contact, various proteases (not shown) concentrate and cleave ECM into smaller fragments, which later are degraded by membrane (MMP14) and soluble (MMP2, MMP9) metalloproteinases. (**D**) The contraction of actin and myosin2 polymer regulated by myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCPtase) induces cell motion. (**E**) Trailing focal contacts detach and disassemble and whole cell body translocates forward, starting a new cycle of movement.



2003]. In our review, we only concentrate on the determined possible interactions between CAs and the proteins that are highly involved in different steps of mesenchymal cell migration.

Most of the cancer cells migrate on ECM by assembling and disassembling specific contacts between the cell membrane and ECM. These contacts are known as FAs [Friedl and Wolf, 2003; Ntantie *et al.*, 2018]. FAs transduce signals from the surroundings of the cell and generate traction forces necessary for cell migration and its regulation [Ntantie *et al.*, 2018]. The adhesomes is a network of multiprotein complexes that coordinates FA signalling between cell and cell or cell and ECM, and it involves around 180 proteins [Zaidel-Bar and Geiger, 2010]. CA IX is a membrane protein, and it is considered to be involved in this signalling network. In addition, CA IX protein-glycan like domain exhibits 38% homology with a keratan-sulphate region of aggrecan, a cartilage protein that can interact

with ECM components hyaluronan and collagen [Opavský *et al*., 1996; Csaderova *et al*., 2013]. This similarity between CA IX and aggrecan supports the idea of possible CA IX involvement in cell–ECM interactions. Moreover, the first steps of FA formation are also influenced by the extracellular pH (pHe) and intracellular pH (pHi) (Figure 2A), which is modulated by many proteins alongside CA IX via its enzymatic activity [Parks, Chiche and Pouyssegur, 2011; Csaderova *et al*., 2013]

Interactions between CA IX and integrins

Assembly of FA sites is a gradual process requiring the systematic recruitment of individual proteins that connect integrins and other ECM receptors with actin cytoskeleton (Figure 2B). Integrins are heterodimeric cell surface receptors that mediate adhesion to the ECM and immunoglobulin superfamily molecules [Desgrosellier and Cheresh, 2010]. The integrin family is composed of 18 α-subunits and 8 β-subunits, which form multiple functional heterodimers that mediate the physical interaction of cells with the ECM [Takada, Ye and Simon, 2007; Cockburn *et al*., 2010]. On ligation to the ECM, integrins cluster in the plane of the membrane and recruit adaptor and signalling proteins. The integrin cytoplasmic tail directly interacts with α-actinin, talin, the FA kinase, and other proteins (Figure 2B) [Friedl and Wolf, 2003]. All these proteins can bind adaptor proteins to recruit actin-binding proteins, as well as regulatory molecules, such as PI3K and RHO-family GTPases [Webb, Parsons and Horwitz, 2002; Banno and Ginsberg, 2008; Csaderova *et al*., 2013].

Swayampakula et al. [2017] showed that CA IX might interact with integrins by proximity-dependent biotinylation (BioID) proteomic analysis and co-immunoprecipitation analyses. Scientists determined that in breast cancer cells MDA-MB-231 overexpressing CA IX, and in wild-type MDA-MB-231 cells under hypoxic conditions, CA IX associates with several adhesion/migration/invasion proteins, particularly integrin-β1 (ITGB1), integrin-α2 (ITGA2) and matrix metalloproteinase-14 (MMP14) [Swayampakula *et al*., 2017]. In the same study, by using immunofluorescent microscopy, authors showed that CA IX co-localises with ITGB1, ITGA2 and MMP14 in actin- and cofilin-positive pseudopodia-like protrusions resembling lamellipodia [Swayampakula *et al*., 2017].

Interactions between CA IX and ROCK1

FAs grow and dissolve in close relation to actin polymerisation and myosin II-generated tension [Friedl and Wolf, 2003; Vicente-Manzanares *et al*., 2009]. RhoA-associated protein kinase (ROCK) is essential for myosin II-generated tension. In addition, ROCK1 is a key signalling regulator of FAs. Activated Rock kinases promote actomyosin contraction (Figure 2D) and the assembly of integrin-mediated FAs [Ntantie *et al*., 2018]. Some data show that specific and non-specific inhibition of ROCK1 or down-regulation of the myosin II activity inhibited FA assembly or enhanced its disassembly [Rottner, Hall and Small, 1999; Pasapera *et al*., 2010; Ntantie *et al*., 2018].

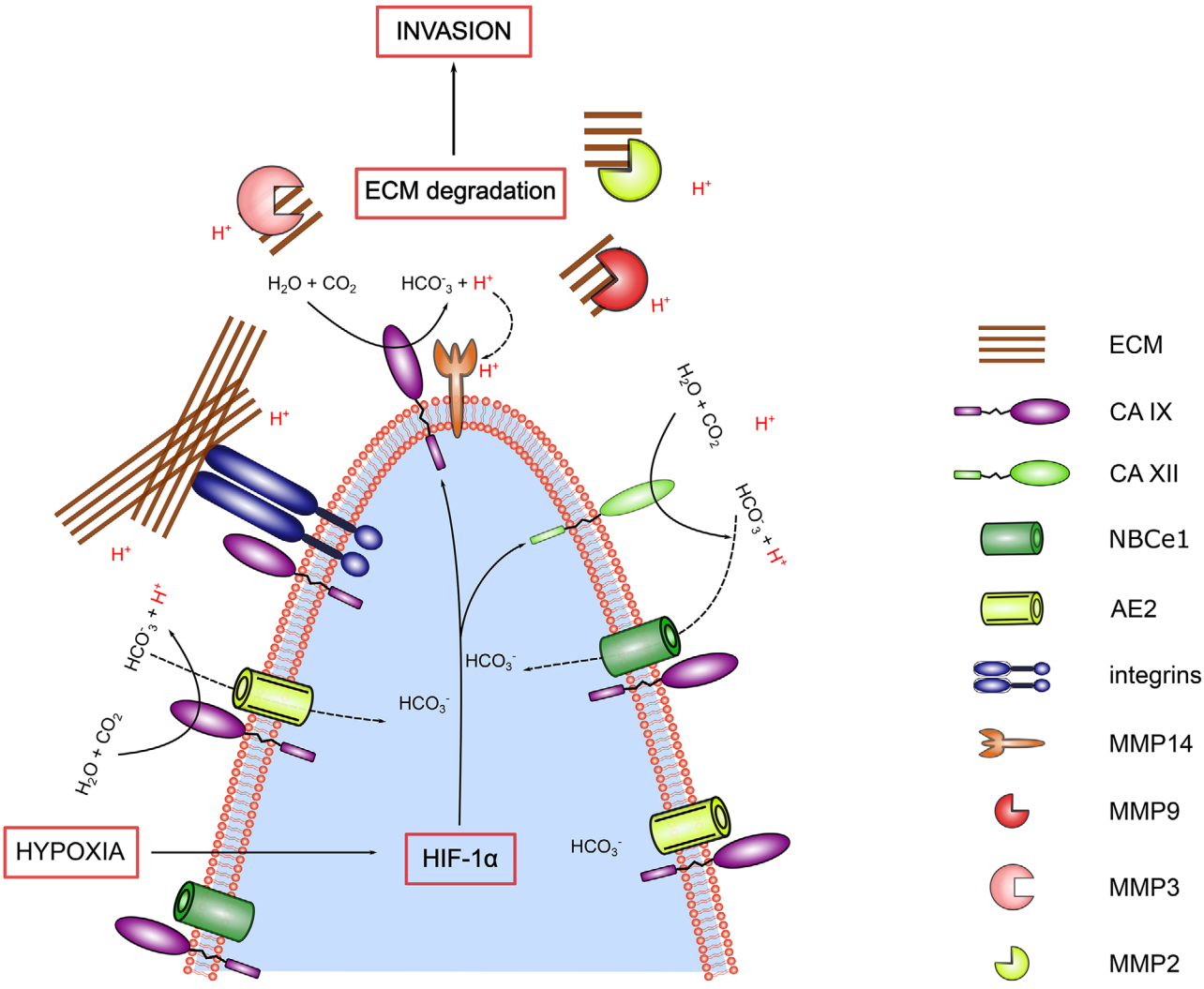
Radvak et al. [2013] in their research demonstrated the connection between CA IX and ROCK1 in HT1080 cells silenced for CA IX. It showed approximately 50% down-regulation of ROCK1 accompanied by the inhibition of FA formation. Csaderova et al. [2013] demonstrated that CA IX silencing decreases ROCK1 levels in both HT1080 and HeLa cell lines. The decreased levels of ROCK1 caused by CA IX silencing resulted in slower cell attachment to the surface, smaller cell spreading area and rare lamellipodia formation, than compared with the parental cell lines [Csaderova *et al*., 2013]. To support the role of CA IX in cancer cell FA process, Csaderova et al. [2013] reported that human cervix carcinoma (C33) cells transfected with CA IX had increased cell spreading area, which led to improved cell attachment to the ECM structures. In the same study, it was showed that CA IX co-localises with paxillin during the formation of a new FA.

Formation, maturation and disassembly of FAs are very dynamic processes, which accompanies cell migration and modulates its speed. All these data support the direct role of CA IX in FAs of cancer cells.

Interactions between CA IX and ion transporters during cell migration

It is already well known that cells exposed to low pHe values show increased invasion and migration both *in vitro* and *in vivo* [Parks, Chiche and Pouyssegur, 2011; Webb *et al*., 2011; Estrella *et al*., 2013]. Cell migration depends on the correct pH gradient along the longitudinal cell axis – with acidic pHe and alkaline pHi at the cell front, and alkaline pHe and acidic pHi at the rear end [Martin *et al*., 2010; Svastova and

Figure 3 | CA IX and CA XII associations with cell membrane structures during hypoxia-induced cell invasion
CA IX co-localises with collagen- and laminin-binding integrins in invadopodias at focal adhesion areas. CA IX couples with NBCe1 and AE2 membrane ion transporters to facilitate influx of HCO^{-}_{3} to maintain pH gradient. CA IX co-localises with MMP14 and facilitates its activity by providing H^{+} after CO_2 hydrolysis. In addition, CA IX and CA XII associate with secreted forms of MMPs. CA IX potentiates the activity of MMP3 by donating extra H^{+}. Moreover, both CAs up-regulate the secretion of MMP9 and MMP2, though the mechanism is yet to be determined.



Pastorekova, 2013; Corbet and Feron, 2017]. In addition, an intracellular pH higher than 7.2 increases the assembly of new actin filaments and a formation of membrane protrusions [Srivastava *et al.*, 2008]. The establishment of pH gradient is associated with re-localisation of ion transporters, including sodium hydrogen transporter (NHE1), sodium bicarbonate co-transporter 1 (NBCe1) and anion exchanger (AE2). They re-localise to lamellipodia (Figure 2A) or to invadopodia (Figure 3) [Schwab *et al.*, 2012; Svastova and Pastorekova, 2013; Stock and Schwab, 2015], where they possibly interact with CAs [Svastova *et al.*, 2012; Swayampakula *et al.*, 2017].

Svastova et al. [2012] reported that in the leading edge of the migrating cells, CA IX co-localises and directly interacts with NBCe1 and AE2. The authors determined these interactions using proximity ligation assay, which allows for detection of

stable transient protein-protein interactions *in situ*. Another group of scientists determined that CA IX increases the activity of Cl^-/HCO_3^- anion exchanger (AE1, AE2, AE3), suggesting its functional interaction with AEs [Morgan *et al*., 2007]. In addition, the same group reported that the catalytic domain of the CA IX coordinates the interaction between the CA IX and AE2, as only the depletion of this domain decreases the activity of AE2 [Morgan *et al*., 2007]. Swayampakula et al. [2017] support the idea of interaction between CA IX and ion transporters, as they performed BioID assay, demonstrating the robust association between CA IX and NBCn1 transporter. In another study, Ditte et al. [2011] reported that CA IX co-localised with NBC1 transporter at the leading edge of the membrane of hypoxic A549 cells, suggesting that there is a strong association between these proteins used to achieve directed ion transport. Debreova et al. [2019] showed that CA IX co-localised with NBCe1 in the invadopodia of HT1080 cells. Parks and Pouyssegur [2015] in their study demonstrated that genetic inhibition of NBC1 in MDA-MB-231 cells resulted in decreased expression of CA IX, supporting the possible association between CA IX and NBC1 transporters [Parks and Pouyssegur, 2015].

Current data strongly suggests that CA IX interacts with ion transporters and it is involved in the formation of pH regulatory metabolon at the leading edge of migrating cancer cell membrane. Interaction between CA IX and bicarbonate transporters maximises the rate of bicarbonate transport across the plasma membrane and increases the pHi in lamellipodia, contributing to pH gradient establishment.

Interactions between CAIX, CAXII and matrix metalloproteinases during cell invasion

Invadopodia is an actin-rich cell membrane protrusion, which breaches the basal membrane and leads to cell invasion [Jacob *et al*., 2016]. The formation and progression of invadopodia have been reviewed elsewhere [Murphy and Courtneidge, 2011]. The matrix degradation activity of invadopodia is attributed to the targeted section of matrix degrading enzymes such as matrix metalloproteinases (MMPs) and pH regulating ion transporters [Stock and Schwab, 2015; Jacob *et al*., 2016]. MMPs belong to the family of zinc-dependent endopeptidases. During cancer progression, MMPs are very important for the local invasion process since they contribute to the loss of the basement membrane barrier through active proteolysis [Jacob *et al*., 2016]. The main MMPs involved in the process of cancer metastasis are MMP2, MMP9 and MMP14 [Clark and Weaver, 2008; Liu *et al*., 2009; Jacob *et al*., 2016].

Some data show that CA IX and CA XII interact directly and indirectly with the MMPs. Hsieh et al. [2010] demonstrated that genetic inhibition of CA XII in breast cancer cells (MB-MDA-231) resulted in decreased expression and activity of MMP2, MMP9 and u-PA, which all take a part in ECM degradation during migration and invasion (Figure 3). Nevertheless, the knockdown of CA XII induces the expression of TIMP-2 and PAI-1, which are the specific endogenous inhibitors of MMP2 and u-PA [Hsieh *et al*., 2010]. Scientists suggest that CA XII might regulate the activity of MMP2 and MMP9 through p38 MAPK pathway, as the knockdown of CA XII resulted in lower levels of phosphorylated (activated) p38 MAPK [Hsieh *et al*., 2010].

There are more data of CA IX and MMPs interactions, as the CA IX is more widely investigated, than compared with CA XII. Radvak et al. [2013] in their study demonstrated that the knockdown of CA IX decreased the expression MMP9, and the activity of MMP2 and MMP3 during cancer cell migration and invasion through matrigel under hypoxia conditions (Figure 3). It is possible that inhibition of CA IX decreases extracellular levels of protons, which are necessary for the protonation of MMP3 and fibrinogen, the substrate of MMP2. Therefore, despite the increased levels of these MMPs, the activity of MMP2 and MMP3 decreases [Rupp *et al*., 2008; Radvak *et al*., 2013]. The down-regulation of MMP9 in CA IX depleted cancer cells can be influenced by reduced levels of ROCK1 [Csaderova *et al*., 2013], as it was determined that inhibition of ROCK1 results in MMP9 down-regulation [Turner *et al*., 2005].

A recent study has demonstrated direct interaction of CA IX and MMP14 (Figure 3) [Swayampakula *et al*., 2017]. In this study, scientists determined that hypoxia-induced CA IX co-localises with MMP14 at mature invadopodia in sites of ECM degradation during cancer cell invasion, determining a possible mechanism of CA IX and MMP14 interaction. Scientists suggested that CA IX could regulate cancer cell invasion through matrigel via its' intracellular and proteoglycan-like domains. Though, the only

deletion of intracellular domain reduced the catalytic activity of CA IX and the invasion through the layer of collagen 1, which is the major component of tumour stroma [Swayampakula *et al*., 2017]. In addition, it is possible, that CA IX could interact with MMP14 directly and indirectly CA IX interacts with MMP14 directly through short regions in the intracellular domain by phosphorylation-dephosphorylation events, and indirectly through its conversion of CO_2 to bicarbonate and H^+, which are then provided to MMP14 [Swayampakula *et al*., 2017].

Available data demonstrate that both CA IX and CA XII are associated with MMPs functionally and in some cases physically, and support the idea of CAs involvement in cancer cell migration and invasion. However, the exact mechanism of CAs role in cancer cell migration and invasion is not yet determined, but associations between CAs and MMPs could provide better insights onto the matter.

The role of CA IX and CA XII in tumour formation

As mentioned earlier, the inhibition of CA IX and CA XII has a serious effect on various cancers cell adhesion, migration and invasion. Therefore, the fact that these transmembrane CAs are involved in cancer cell migration, adhesion and invasion, and are highly expressed mostly by cancer cells, makes these CAs an intriguing target for anti-metastatic therapy.

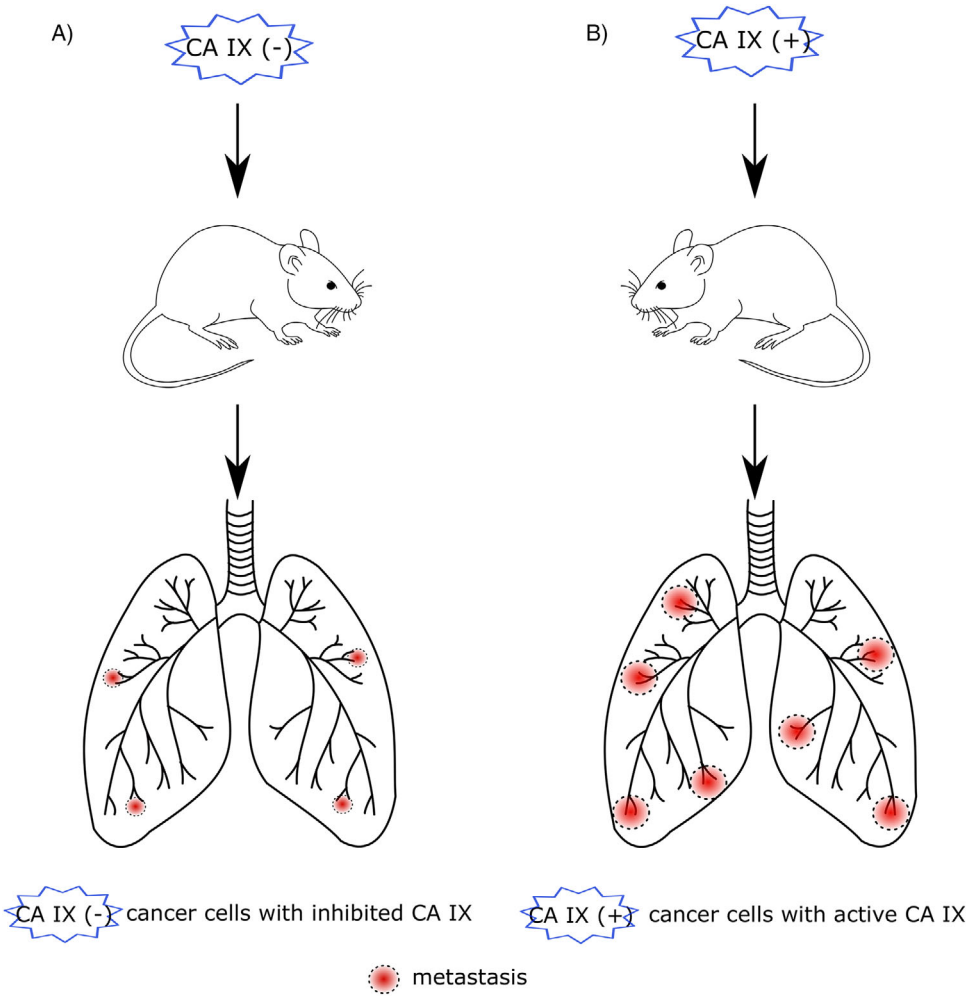
Chiche et al. [2009] was the first group of scientists to report CA IX and CA XII role in tumour growth both *in vitro* (tumour spheroids) and *in vivo* (nude mouse models). The group demonstrated that colon adenocarcinoma (LS174Tr) cells genetically silenced for CA IX (Figure 4A) or both CA IX and CA XII, formed smaller spheroids *in vitro* under hypoxic conditions and resulted in a decreased rate of tumour growth in nude mouse model, than compared with the normal LS174Tr (Figure 4B) cells. However, depletion of CA XII alone in cells had no significant effect on the spheroid size or the rate of tumour growth in mice [Chiche *et al*., 2009; Chen *et al*., 2018]. Also, there is some evidence that knockdown of CA IX gene in cancer cells might increase the expression of CA XII [Chiche *et al*., 2009; McIntyre *et al*., 2012], which could possibly appear as a compensatory mechanism for maintaining pHi under hypoxic conditions. Therefore, the higher antimetastatic effect could be achieved through the inhibition of both CAs. Another study showed that CA IX expression was associated with increased cell necrosis in tumours, as an introduction of CA IX to HCT116 cells increased cell necrosis in spheroids and xenografts, and the knockdown of CA IX in HT29 cells decreased cell necrosis in spheroids and xenografts [McIntyre *et al*., 2012]. Scientists suggested that increased necrosis in tumours was a result of an increased tumour volume. In addition, they hypothesised that necrosis might represent regions where CA IX had maintained a high level of proliferation through maintaining more alkaline pHi [McIntyre *et al*., 2012].

The role of CA IX and CA XII in tumour metastasis

According to summarised literature (Table 1), genetic or pharmacological inhibition of CA IX in combination with anti-angiogenic or anticancer agent decreased the growth of xenograft tumours in nude mice more efficiently, than compared with a single agent treated mice [McIntyre *et al*., 2012; Lock *et al*., 2013]. Also, Lock et al. [2013] reported that fewer and smaller metastases were formed in mice injected with MDA-MB-231 cells after treating them with a combination of specific CA IX inhibitor U-104 and anticancer agent paclitaxel, than compared with single-agent therapy. The same group of scientists determined that the inhibition of CA IX in tumour cells resulted in inhibition of the mTORC1 signalling pathway, as CA IX inhibition reduced phosphorylation of ^{Ser939}TSC2, inhibiting mTORC1 signalling [Lock *et al*., 2013]. These results provide better insights of CA IX role in mTOR signalling in cancer cells *in vivo*. Swayampakula et al. [2017] also reported that CA IX might have an important role in cancer metastasis, as 4T1 cells with the knocked down of CA IX and mutant 4T1 cells, expressing CA IX with truncated intracellular or proteoglycan-like domains formed fewer metastases in mice lungs after tail vein injection compared to control 4T1 cells. To support the hypothesised role of CA IX in metastases formation, the same group of scientists demonstrated that reintroduction of CA IX in cancer cells resulted in similar levels of lung metastases in mice compared with the normal 4T1 cells [Swayampakula *et al*., 2017].

Figure 4 | The role of CA IX in metastases formation

Pre-clinical data demonstrates that in mice injected with CA IX, the cancer cells were inhibited/knocked-down, and therefore, the chances of metastases are reduced (**A**), compared with the mice injected with CA IX positive cancer cells (**B**).



The data on CA XII role in cancer metastasis are contradictory. As mentioned earlier, Chiche et al. [2009] demonstrated that the knockdown of CA XII did not have any significant effect on adenocarcinoma metastases development in mice. Meanwhile, another group of scientists has demonstrated that after the knockout of CA XII, MDA-MB-231 cells developed fewer tumour metastases which were smaller in size, than compared with the control animals [Hsieh *et al.*, 2010]. However, the number of experimental data on CA XII role in tumour metastases is limited, and therefore, it is not possible to draw any conclusions.

The clinical data on CA XII correlation to patient prognosis is also contradictory. Some studies have reported that CA XII is a good prognostic marker in patients with invasive breast cancer [Watson *et al.*, 2003] or with respectable non-small cell lung cancer [Ilie *et al.*, 2011], whereas in another study, CA XII has been reported to be a poor prognostic marker for patients with astrocytomas [Haapasalo *et al.*, 2008].

The *in vivo* data show that CA IX is highly involved in metastatic tumour formation and growth of various cancers, as the involvement of CAXII in cancer metastasis is still contradictory.

Table 1 | Summary of pre-clinical data on CA IX and CA XII role in cancer metastasis

CA	Cancer type (cell line)	Design of research	Results	References
CA IX and CA XII	Colon adenocarcinoma (LS174Tr)	Knockdown cells for CA IX, CA XII and CA IX/CA XII injected to mice	Cells knocked-down for CA IX/CA XII and CA IX decreased tumour growth rate, as knockdown of CAXII had no effect on tumour growth	Chiche *et al*. [2009]
CAIX	Human colon cancer (HT29); colorectal carcinoma (HCT116)	HT29 cells knocked-down for CA IX and HCT116 cells with introduced CA IX injected to separate groups of mice;	Knockdown of CA IX in HT29 cells reduced tumour growth rate; Introduction of CA IX to HCT116 increased tumour growth compared with the control groups	McIntyre *et al*. [2012]
CAIX	Human colon cancer (HT29)	Pharmacological inhibition of CA IX with acetazolamide and/or Bevacizumab	Pharmacological inhibition of CA IX with acetazolamide in combination with Bevacizumab significantly reduced xenograft tumour growth rate	McIntyre *et al*. [2012]
CAIX	Murine breast cancer (4T1)	Knocked-down for CA IX cells injected to mice	4T1 CA IX(-) cells formed less metastases	Swayampakula *et al*. [2017]
CAIX	Human breast cancer (MDA-MB-231); murine breast cancer (4T1)	Pharmacological inhibition of CA IX with CA X-specific inhibitors and/or paclitaxel	After treating with CAIX inhibitors alone, and in combination with paclitaxel, less metastases formed in mice	Lock *et al*. [2013]
CAIX and CAXII	Human breast cancer (UFH-001)	UFH-001 cells knocked-down for CA IX or CA XII injected to mice	UFH-001 CA IX(-) formed less metastases compared with UFH-001 CA XII(-)	Chen *et al*. [2018]
CAIX	Human breast cancer (MDA-MB-231)	Pharmacological inhibition of CA IX	Decreased tumour growth rate and metastases size in mice	Ward *et al*. [2015]
CAXII	Human breast cancer (MDA-MB-231)	Cells knocked-down for CA XII injected to mice	Reduced metastases number and size in mice compared with the control group	Hsieh *et al*. [2010]

Conclusions and clinical perspectives

Available *in vitro* and *in vivo* data have demonstrated that genetic or pharmacological inhibition of CAs decreases various steps of metastasis processes. In addition, *in vitro* data demonstrate that CA IX might interact with E-cadherin connections, decreasing cell–cell adhesion, and therefore, increasing individual cancer cell motility. Nevertheless, CA IX co-localises and interacts with various membrane proteins, such as integrins (α, β), matrix metalloproteinases (MMP14) and ion exchangers (NBCe1, AEs), which are involved in cancer cell migration and invasion processes. Also, there is some evidence that both CA IX and CA XII interact with extracellular MMPs (MMP2, MMP3, MMP9) which are the key proteins to degrade ECM. *In vivo* data demonstrated that genetic or pharmacological inhibition of CA IX or CA XII or both could decrease the formation of metastases in number and in volume, though the data on CA XII was quite controversial. The data discussed in this review highly supports the possible role of CAs in various cancer metastasis processes, though the exact mechanisms of interactions between CAs and other proteins involved in cancer metastasis are not yet determined and need further investigation.

CA IX and CA XII are interesting not only as research subjects but also as subjects with a potential clinical application. Recent studies have shown that both proteins could be used as the therapeutic or diagnostic targets, mainly for the management of hypoxic tumours and metastases [Gieling *et al*., 2012;

Lounnas *et al*., 2013; Kopecka *et al*., 2015; Tafreshi *et al*., 2016]. Small molecule compounds or monoclonal antibodies targeting CAs have already been proven to inhibit cancer cell proliferation and formation of metastases in several xenograft mice models [Lou *et al*., 2011; Gieling *et al*., 2012; Chen *et al*., 2018]. Several studies have demonstrated that targeting CA XII could help to overcome chemoresistance of cancer cells [Ward *et al*., 2015; Kopecka *et al*., 2016; von Neubeck *et al*., 2018]. In addition, small fluorescent molecular compounds targeting CA IX, or monoclonal antibodies conjugated with fluorescent probes and targeting CA IX, have been successfully used for metastatic breast cancer imaging in xenograft mice models [Tafreshi *et al*., 2012, 2016].

All discussed information on CA IX, CA XII and their involvement in cancer cell migration, invasion and metastasis are quite recent, and there is still a lot to investigate. Nevertheless, all findings show the great potential of these CAs in the context of research and application in clinical use.

Conflict of interest statement

The authors have declared no conflict of interest.

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