Carbonic Anhydrase IX Shedding Through Exosomes in Renal Carcinoma Cells

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Abstract: Carbonic anhydrase IX (CA IX) is a transmembrane glycoprotein highly expressed in clear cell carcinoma of the kidney and in a wide variety of other human cancers. Its expression is largely under the control of intratumoral hypoxia and in some subtypes of renal cell carcinoma (RCC), by the presence of a defective version of the von Hippel Lindau (VHL) gene by which the HIF-1α factor is stabilized. Several notable studies have shown that CA IX is involved in the control of acidity intracellularly. Thus, this enzyme could be involved in the regulation of pH within the tumor and may modulate the tumor microenvironment. Recent studies have also shown that this enzyme could be involved in the control of cell adhesion processes between the cancer cells, contributing to their invasive potential. Here, we have analyzed the effect of increasing the level of CA IX expression in the CA IX negative SKRC-17 renal cancer cells. Our results showed that CA IX is recruited to the lipid rafts of these cancer cells. Our results also highly suggest that the lipid raft component gangliosides are shed in the form of exosomes in this model system as a result of forced CA IX expression. The level of ganglioside shedding positively correlated with the increased level of CA IX expression in these cells. Finally, from a glycobiological perspective, our results indicate that elevated levels of CA IX expression contributes to increased ganglioside shedding in the form of exosomes which in turn may contribute to increased invasiveness and immunological escape in this malignancy.

Keywords: carbonic anhydrase IX, renal carcinoma, exosomes, gangliosides
Introduction
Renal cell carcinoma (RCC) accounts for roughly 2% of all cancers, with the highest rate of occurrence within the United States and Northern Europe.1,2 RCC is well known clinically for its lack of early warning signs, resulting in a significant occurrence of metastatic disease progression at first diagnosis. Recent advances in our understanding of the molecular pathogenesis of RCC, greatly aided by the introduction of the cDNA microarray technology, have provided a clearer picture of the underlying molecular and genetic alterations in RCC.3,4 Currently, RCC is viewed not as a single entity, but as a mixture of several sub-types of diseases with different biological attributes and unique molecular signatures that happen to occur in a single organ, i.e. the kidney.5,6 Extensive biochemical analyses including the molecular profiling of these RCC subtypes revealed the association of a class of Carbonic Anhydrase isoenzymes (CA IX and CA XII) which are membrane bound zinc metalloenzymes, with clear cell carcinoma of the kidney.7-9 Initial studies focused on their role as a molecular marker since their expression is mostly cancer restricted, the only exception being their normal expression in the dissociated epithelial cells lining the gastrointestinal tract and pancreas.10-12 For clear cell carcinoma of the kidney, the CA IX protein appears to play a significant role in the adaptation to an hypoxic environment and may be involved in the tumor progression since its expression is not seen in the normal renal tissue.13,14 CA IX is a novel member of the phylogenetically well conserved carbonic anhydrase family.15,16 It is a single span transmembrane glycoprotein that possesses an extracellular catalytic domain which is preceded by a novel proteoglycan domain (PG domain). It catalyzes the reversible hydration of CO₂ (CO₂ + H₂O ↔ HCO₃⁻ + H⁺). There are 15 iso-enzymes of CA identified so far and these are found in diverse subcellular locales such as the cytoplasm and mitochondria.17 Of these enzymes, CA IX is a membrane bound version and its over-expression on the cell surface is seen in a number of solid tumors, particularly in clear cell RCC and including cervical, ovarian, colorectal, head and neck, bladder and non-small cell lung carcinomas.18,19 In all these malignancies, the expression of CA IX is invariably linked to the development of tumor hypoxia which is mediated by the transcription factor HIF-1. But in the case of clear cell RCC, the CA IX can be expressed not only by the hypoxic regions of tumors but also by the defectiveness of the von Hippel Lindau (VHL) gene by which the HIF-1α factor is stabilized. Other HIF-1 target genes induced by intratumoral hypoxia include glucose transporter-1 (GLUT-1), several glycolytic enzymes and growth factors such as vascular endothelial growth factor (VEGF) which are all factors essential for survival, adaptation to the hypoxic environment, angiogenesis and for the invasive characteristics. Studies with clear cell RCC revealed that the CA IX expression is associated with the mutation(s) in the von Hippel-Lindau tumor suppressor gene (VHL) or promoter suppression by methylation with subsequent loss of pVHL function or expression, apart from intratumoral hypoxia.20,21 CA IX protein can also be over-expressed in RCC clear cell tumors that arise in a sporadic manner.22 In RCC cell lines that over-express CA IX, the expression could be suppressed if the wt VHL gene was re-introduced by transfection.23 Several landmark investigations have elucidated the role of VHL in the regulation of HIF-1α.24-26 Thus, the regulation of CA IX expression and its function as a HIF-1α responsive gene (through VHL independent as well as VHL mediated mechanisms) has made clear cell RCC the most well characterized subtype of all RCCs with serious implications for our understanding of the hypoxic tumor metabolism and the mechanisms by which the tumor cell manages to maintain intracellular pH homeostasis.

Our initial studies showed that this CA IX protein could be multifunctional and is an active participant in the growth factor receptor mediated signaling pathways in renal cancer, particularly that of the epidermal growth factor receptor (EGF-R).27,28 Our studies also identified a positive feedback loop involving CA IX could form the basis for the progression of this cancer and for poor prognosis. We next wished to investigate the role of over-expression of CA IX in renal carcinoma cells from a glycolbiological perspective and understand the role that glycoconjugates, gangliosides in particular, play in the biology of CA IX and its elevated expression in renal cancers. It is well known that certain renal carcinoma cells shed tumor specific gangliosides and this phenomenon is implicated in tumor progression, cell signaling pathways and in
tumor immune escape. Since CA IX is implicated in poor prognosis of several human cancers, we hypothesized that there may exist a relation between CA IX over-expression and ganglioside shedding. We report here our findings which highly suggest that there is a positive correlation between the level of CA IX expression and the level of ganglioside shedding through exosomes in the renal cancer cell line SKRC-17 which is a non-expressor of CA IX to begin with. The implications of our observations on the prognostic significance of CA IX expression in this malignancy are discussed.

Materials and Methods

Cell culture
The SKRC-17 cells which does not express CA IX; SKRC-08 and SKRC-01 renal cell carcinoma (RCC) cell lines which are medium and high expressors of CA IX respectively are a kind gift from Neil Bander (Weill Medical College, Cornell University, NY). These cell lines are routinely maintained as described earlier.

Stable transfection of CA IX into SKRC-17 cells
The CA IX containing plasmid (pSG5-C-CA IX) was a kind gift of Jaromir Pastorek (Institute of Virology, Bratislava, Slovakia). The CA IX negative SKRC-17 cells were transfected with pSG5-C-CA IX and the selection marker plasmid pCNA3.1 exactly as described earlier. As negative control, the empty vector pSG5C and the selection marker plasmid pCDNA3.1 were transfected under the same conditions. Individual colonies were trypsinized and expanded in complete growth medium supplemented with G418 at 500 µg/ml. The expanded colonies were tested for their level of expression of CA IX by immunoblotting and candidate clones were selected for low, medium and high expression of CA IX. G418 resistant colonies were also selected for the isolation of empty vector transfected negative control clones and all were maintained under conditions described earlier.

Isolation of exosomes
Exosomes were isolated from the cell culture supernatant (conditioned medium, CM) by the method of Shedden et al. Briefly, the cancer cells were grown to 50% confluency. At that time, the medium was replaced with fresh medium and the cells were allowed to grow to 100% confluency, which took approximately 48 hours. Conditioned medium (CM) was collected. CM was centrifuged at 1000 g for 10 minutes to remove any floating cells or debris. The CM supernatant was then centrifuged at 100,000 g for 1 hour. The exosome vesicle pellet (UP, ultracentrifuge pellet) and the supernatant (ultracentrifuge supernatant, US) fractions were collected. The US fraction was further concentrated by an Amicon ultrafiltration unit and the protein contents were estimated and normalized by a DC protein assay kit (BioRad), before subjecting the fractions to western blot analysis, using a monoclonal antibody against CA IX (clone M75, Bayer health care Division) as described earlier. The signals were detected using the enhanced chemiluminescence method using the horse radish peroxidase conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnologies, Inc), according to the manufacturer’s instructions (Amersham, Inc).

Recruitment of CA IX into lipid rafts
Crude membranes and lipid rafts were isolated by the single and short density gradient centrifugation method of MacDonald and Pike. Briefly, crude membranes were isolated by suspending the cells (SKRC-01, 08 and 17) at 4°C in the base buffer of 20 mM Tris HCl pH 7.8 with 250 mM sucrose, in the presence of 1 mM MgCl$_2$ and 1 mM CaCl$_2$ and in the presence of the protease inhibitor cocktail (Roche). The cells were lysed by passage through a 27 gauge needle, for 20 times. Lysates were then centrifuged at 1000 g for 10 minutes. The resulting post nuclear and debris free supernatant was ultracentrifuged at 100,000 g for 60 minutes to get the crude membrane pellet fraction. For the isolation of the lipid rafts, the carbonate single step gradient method of Song et al was used. Briefly, the cultured kidney cancer cells were washed and scraped with ice cold phosphate buffer saline (PBS). After pelleting the cells for 2 min at 1000 g, 2 ml of 0.5 M sodium carbonate, pH 11.0 containing a cocktail of protease inhibitors (as above) was added to the cell pellet and lysed in a Dounce homogenizer with a tight fitting pestle. This cell lysate was further lysed by passage through a 27 gauge needle for 10 times. Finally, the cell lysate was sonicated in a Branson probe type
sonicator for 30 seconds. The homogenate (2 ml) was mixed with an equal volume of MES-saline-sucrose (25 mM MES, pH 6.5 and 150 mM NaCl containing 90% sucrose) and placed at the bottom of the ultracentrifuge tube. Four milliliters of the MES-saline containing 35% sucrose was layered on top followed by 4 ml of MES buffered saline containing 5% sucrose. Gradients were centrifuged for 16 hrs at 175,000 g in a swing-out bucket rotor. The tubes were fractionated into 24 half ml fractions. The lipid raft fractions that appear at the interface 5 and 35% sucrose cushions are collected and used for further analyses in western blots. SKRC-17 renal cancer cells that do not express CA IX are used as a control throughout these studies. SKRC-08 cells are medium and SKRC-01 cells are high expressors of CA IX protein, respectively. Flotillin antibody was used to probe the western blots as a marker for both the crude membrane as well as the lipid raft fractions that were isolated after sucrose density gradient centrifugation.

Total lipid bound sialic acid assay (LBSA)
The control untransfected SKRC-17 cells and the medium and high CA IX expressing SKRC-17 clones are expanded in cell culture. For the estimation of total lipid bound sialic acid, which is a measure of the ganglioside content of these cells, the original method of Ladisch and Gillard was adapted. Briefly, the trypsinized cells (10⁷ to 10⁸ cells) were washed three times in the serum free medium and centrifuged at 1000 g for 5 minutes. The cell pellet was lyophilized and extracted with chloroform: methanol (C:M, 1:1, 20 vol/gm of cells). The solids were dispersed by sonication for 3 minutes. The sonicate was extracted for 18 hrs at 4 °C with gentle magnetic stirring. The tubes were spun at 1000 g for 10 minutes and the clear supernatant was decanted. The remaining pellet was re-extracted with fresh C:M 1:1. The extract derived from this step was mixed with that of the first step and cooled overnight at −20 °C to precipitate and remove any proteins, particularly glycoproteins. The cooled extract was re-centrifuged as above to recover the total lipid supernate. An aliquot of this total lipid extract was lyophilized and treated through the original method of Svennerholm, as described by Chu et al. for the estimation of total sialic acid by the resorcinol assay. The final results were described as nanomoles of lipid bound sialic acid per 10⁸ cells.

Results and Discussion
CA IX is recruited to the lipid rafts
Figure 1 shows the differential distribution of the integral membrane protein CA IX in crude membrane and in the isolated lipid rafts in immunoblots using

![Figure 1](image)

**Figure 1.** CA IX is recruited to lipid rafts in SKRC cells. Three cell lines were employed, namely the CA IX negative SKRC-17 cells, and the CA IX (+) SKRC-08 cells and the CA IX (+++) SKRC-01 cells. This Figure shows the differential distribution of the integral membrane protein CA IX in crude membrane and in lipid rafts isolated from these cells in immunoblots using the mouse monoclonal antibody against CA IX (M75) whose epitope resides in the proteoglycan domain (PG domain). While the isolated crude membranes reveal the presence of both the intact (54 KDa) as well as the cleaved 49 KDa CA IX proteins, the lipid rafts harbor only the intact protein (upper panel). Flotillin expression (45 KDa) was followed in parallel immunoblots as a marker for lipid raft preparations as well as crude membranes (lower panel). Normalized amounts (10 micrograms) of protein was applied to each lane.
the mouse monoclonal antibody against CA IX (M75, Bayer Healthcare Division), whose epitope resides in the proteoglycan domain (PG domain) of CA IX. While the isolated crude membranes reveal the presence of both the intact (54 KDa) as well as the cleaved 49 KDa CA IX proteins, the lipid rafts show only the intact protein. But, in the purified membrane preparations, both the intact and the cleaved fractions are observable. These results highly suggest that a form of ectodomain cleavage occurs on the cell membrane by a specific protease. Recent work of Pastorek and coworkers revealed that this surface protease might be TACE (TNF-α converting enzyme, also called ADAM-17, a disintegrin and metalloprotease). Their work also revealed that the cleavage of CA IX occurs at a putative site at or very close to TM (transmembrane) domain. This is inferred since the M75 antibody recognizes both the intact and the cleaved versions of CA IX protein, and the cytoplasmic tail has a molecular weight of around 5 KDa. If the surface proteolysis had occurred in any other way, the proteoglycan domain would have been cleaved and the epitope lost and hence the remaining protein would not have been recognized by the M75 antibody.

**CA IX is present in isolated exosomes**

Figure 2 shows the localization of both the intact as well as the cleaved CA IX proteins in the exosome (lane 4, 01 UP fraction). Whereas, in lanes 2 and 3, the relative proportion of the cleaved CA IX is high and it predominates over the intact version (both in the conditioned medium and in the supernatant), whose signal is negligible on the immunoblot. The exosomal pellet (UP) fraction, present in a concentrated pellet form allows one to visualize the presence of both the intact as well as the cleaved fractions of the protein (lane 4). An aliquot of the total cell lysate, shown in lane 1 as a control, reveals the presence of the intact protein only, very likely due to a disproportionate representation of the cleaved protein. Thus, the CA IX protein (both the intact as well as the cleaved versions) can be differentiated in the lipid rafts and exosomal vesicles depending upon where they are located. It is very likely that the cleaved CA IX represents a very minute fraction in the whole cell lysate which could not be detected in the immunobLOTS (Figure 2).

As another negative control, the CA IX negative SKRC-17 cells were fractionated in an identical manner, as shown in lanes 5, 6 and 7, showing non-specific cross reacting bands. These preliminary findings with respect to the hypoxia inducible Carbonic Anhydrase IX enzyme are significant for the following reasons. These studies show that there is a close relationship between the localization of CA IX protein in the lipid rafts (at or very close to where the TACE or other sheddases are located) and the genesis of exosomes. There are several examples in the literature that highly suggest the presence of the lipid raft microdomains in the exosomal membranes and suggest their participation in exosome vesicle membrane formation and structure. These findings suggest, at the least in a preliminary

![Figure 2](image-url). Isolation of exosomes and the localization of both intact and cleaved CA IX in these vesicles in CA IX negative SKRC-17 as well as CA IX (++++) SKRC-01 renal carcinoma cells. See text for details of the isolation procedure. CM: conditioned medium; US: ultracentrifuge supernatant; UP: ultracentrifuge pellet. Lane 1: whole cell lysate from the CA IX positive SKRC-01 cells. The cleaved version is not visible in the whole cell lysate, where the intact CA IX predominates. In lane 2 and 3 (CM and US) the cleaved version predominates. The exosome pellet fraction (UP, lane 4) allows the visualization of both the intact and the cleaved versions of CA IX (54 KDa and 49 KDa). Higher molecular weight versions (approx 80 KDa) of non-specific cross reacting proteins were observed in the conditioned media, supernatant and pellet versions of both SKRC-17 and SKRC-01 cells and hence were not considered (see Figure).
way, that exosomes may be involved in regulatory mechanisms, i.e. in the regulation of intacellular pH. Whether they are involved in the regulation of extracellular pH, is not currently known. Moreover, Zavada and coworkers reported earlier that a soluble form of Carbonic anhydrase IX (CA IX) is present in the serum and in the urine of renal carcinoma patients. A close examination of the sizes of the intact and the cleaved versions of CA IX in the serum and in urine, using western blots reveal a putative cleavage site of CA IX, which correlates with the findings described above as well as the conclusions reached by Pastorek’s group that the cleavage occurs at or very close to the TM domain. The most important finding as presented in these preliminary experiments is that both the intact and the cleaved versions of CA IX could be seen in the shed exosomal vesicles whereas the lipid raft microdomains harbor mostly the intact protein. These findings also imply that while the intact CA IX may be involved in the robust regulation of intracellular pH (pHi, through a possible formation of a metabolon complex with the intracellular CA II and the sodium ion/bicarbonate exchanger) of the cancer cell, the exosomally located CA IX may be relieved of these constraints as it is shed out extracellularly and hence it is now in a position to modulate the extracellular matrix and the tumor microenvironment through the free and mobile extracellular proteoglycan domain and possibly participate in the regulation of the activity of several cell surface proteases such as matrix metalloproteinases (MMPs) and ADAMs. These hypotheses are currently under active investigation. Thus, a study of the exosomally located CA IX may yield invaluable clues as to the stage, progression and the molecular mechanisms of this disease. Presence of CA IX in the extrosomal fraction of urine or serum may well be an early bio-marker that may offer poor prognosis. However, these hypotheses need to be verified by a proteomic analysis of these exosomal vesicles, with particular reference to the presence and activity of CA IX on these vesicles with a proper correlation with the pathologic aspects of the disease.

CA IX expression positively correlates with increased ganglioside shedding in SKRC-17 renal carcinoma cells

Our preliminary findings suggested that increased ganglioside shedding might occur as the level of CA IX expression was increased. We postulated this hypothesis after observing gradually elevated levels of gangliosides in the conditioned medium when the level of expression of CA IX was increased using three different RCC cell lines, namely CA IX –ve SKRC-17, CA IX (++)SKRC-08 and CA IX (++++) SKRC-01 cells. To investigate this hypothesis further, we decided to increase the level of expression of CA IX in the same cell line system, using the CA IX –ve SKRC-17 cell line and investigate the hypothesis whether elevated levels of CA IX expression allows for the increased shedding of gangliosides in the same cell system as background. To this effect, we stably transfected SKRC-17 cells with CA IX containing plasmid exactly as described by Svastova and coworkers. The results are shown in Figure 3 which describes the isolation of high (H), medium (M) and low (L) expressors of CA IX. The amount of total cellular ganglioside is comparable between the control plasmid (pSG5C and pCDNA 3.1 only) transfected cells and the CA IX medium expressors and the CA IX high expressor clones, when the gangliosides are expressed as total lipid bound sialic acid (LBSA). Interestingly, we observed a significant increase in the level of shed gangliosides in the exosome fractions prepared from the conditioned medium (CM) from these CA IX expressing clones, as compared to the control transfected clones (Table 1).

Biological significance of these findings

In conclusion, the integral membrane enzyme CA IX is over-expressed in several renal cell carcinoma cell lines and in tumors and is associated with disease progression, more so in the early stages of this malignancy, possibly due to contrasting biphasic expression
levels of HIF-1α and HIF-2α, unlike other cancers such as colorectal cancer. Nevertheless, CA IX is expressed in the carcinoma cells in a truncated soluble form. Truncated CA IX is also present in serum as well as urine of kidney cancer patients. We have used several kidney cancer cell lines expressing CA IX to analyze the constitutive cleavage of the protein by immunoblotting procedures. We find that in kidney cancer cells, the constitutive cleavage of CA IX proceeds in secretory vesicles known as exosomes. Since this protein is known to be a hypoxia (HIF-1α) inducible marker, it is entirely possible that hypoxia enhances the ectodomain shedding of CA IX in these exosomal vesicles from renal cell carcinomas and in other cancer systems. Thus, the detection of CA IX in these vesicles in serum may suggest the presence of severely hypoxic regions in the tumor and offer a means for tumor detection as well as poor prognosis. While TACE/ADAM-17 is known to play a role in the shedding of the cleaved form of CA IX in exosomal vesicles, participation of other sheddases (membrane proteases) can not be ruled out. Further characterization is needed with respect to specific markers for these exosomal vesicles, particularly with respect to how CA IX relates to or interacts with the other exosomal protein complexes such as sorting proteins ESCRT-1, -2 and -3. Also, whether CA IX participates in the segregation of other cargo proteins such as galectins, microRNAs and other specific glycolipids and how CA IX participates in the multivesicle body (MVB) formation leading to exosome biogenesis is still unclear. CA IX is known to be phosphorylated by Akt and to participate in the phosphoinositide signaling. It is also known that the final steps in the MVB formation involves lipid metabolism and phosphoinositide signaling. Thus, it becomes necessary to investigate whether there is a correlation here in order to understand the role of CA IX in exosomal shedding in these cancer cell lines as well as renal cancers in vivo.

From a glycobiological perspective, the principal finding of our studies is the observation that ganglioside shedding increases in a renal cell carcinoma cell system as a result of elevated CA IX expression. Since elevated ganglioside shedding or exogeneous addition of gangliosides are known to contribute to elevated src kinase activity, it is not unreasonable to speculate that elevated CA IX expression contributed, possibly by both direct and indirect pathways, to elevated src activity in our cell system. Indeed, we have observed in our CA IX transfected cell system a significant elevation of the src tyrosine kinase activity (data not shown). It is possible that renal cancers which often grow to become large masses in vivo have regions of hypoxia in their cores. Since CA IX is one of the target genes of hypoxia inducible factor-1α (HIF-1α), it is entirely possible that, on the basis of our results, regions of hypoxia in tumors may shed more gangliosides than what occurs in non-hypoxic regions and possibly contribute to more aberrant growth factor receptor and src signaling pathways. It is also interesting to note that several renal cell carcinomas (belonging to clear cell, papillary and the chromophobe varieties) shed specific gangliosides as shown by the work of Hakomori and others. Thus, the qualitative and quantitative aspects of this phenomenon of ganglioside shedding may offer variety and influence how these growth factor receptor and the src signaling pathways are modulated in a particular cancer.

It is known that tumors actively release exosomes and participate in immunosuppressive processes in their microenvironment, it is possible that these gangliosides shed in the form of exosomes actively contribute to these immunosuppressive mechanisms. Although precise biological function of these exosomes still remain elusive, the tumor derived exosomes and hence the gangliosides and lipid rafts shed as exosomes

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**Table 1.** An analysis of the cellular ganglioside fraction from the total lipid isolates from the control plasmid transfected (pSG5C empty vector and pCDNA3.1 only, neo) and from the clones of SKRC-17 cells selected for medium (+) and high (+++) expression of CA IX. On immunoblots, the level of expression of CA IX in medium expressors and high expressors roughly correlated with the CA IX expression in native SKRC-08 and SKRC-01 cells respectively (data not shown). The ganglioside contents were expressed as total lipid bound sialic acid (LBsA). The LBsA of the shed gangliosides in the conditioned medium is expressed by analyzing the exosome fraction from the CM (UP, see Figure 2).

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<th>Neo + vector</th>
<th>neo + CA IX med</th>
<th>neo + CA IX high</th>
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<tr>
<td>A. Total cellular ganglioside</td>
<td>162 ± 11</td>
<td>170 ± 8</td>
<td>188 ± 13</td>
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<td>B. Shed gangliosides in CM</td>
<td>40 ± 4</td>
<td>113 ± 12</td>
<td>148 ± 15</td>
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**Notes:**
- A: nmole/10⁸ cells, LBsA.
- B: pmole/10⁸ cells/hour, LBsA.

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may influence a series of functional alterations that occur in T-cells in patients with renal cancer and other cancers, ranging from induction of apoptosis and defects in T-cell receptor components and function.\textsuperscript{52–54} These tumor derived exosomes and hence the component gangliosides may also target antigen presenting cells (DC cells) and participate in an upstream blockade of anti-tumor immune responses. Apart from gangliosides and other specific lipids, these tumor derived exosomes express a large array of bio-active molecules such as FasL and TRAIL, through which these vesicles induce apoptosis in activated antitumor T-cells and abrogate the potential of these effector cells to kill tumor cells.\textsuperscript{55} More over, these vesicles can also exert inhibitory activity on T-cell proliferation and skew monocyte differentiation into myeloid suppressive cells. Thus, CA IX overexpression, which contributes to ganglioside and exosome shedding may indirectly participate in the immunosuppressive mechanisms of these renal and other CA IX expressing cancers. Gangliosides, which are integral components of lipid rafts may play an important role in the formation and function of these exosomal vesicles.\textsuperscript{56} These observations are diagrammatically shown in Figure 4.

\textbf{Figure 4.} Schematic representation of the biological significance of over-expression of CA IX and its consequences in renal carcinoma cells from a glyco-biological perspective. This Figure also proposes a hypothesis as to how CA IX and other exosomally associated proteins may participate in the mechanisms of disease progression. Gangliosides which are specific as well as non-specific to the malignancy are localized in the lipid rafts. Carbonic anhydrase IX is also recruited to the lipid rafts where it undergoes ectodomain cleavage with a specific membrane protease (sheddase). The cleaved CA IX as well as a proportion of the uncleaved CA IX undergo internalization, ultimately to be degraded in the lysosomes. Perturbation of this lysosomal fusion impairs this endocytic pathway and hence multivesicular bodies (MVBs) are created. These MVBs undergo fusion with the plasma membrane and the exosomes which are marked with gangliosides as well as the partial and intact CA IX are secreted out into the extracellular space. At the least, the intact proteoglycan (PG) domain of the shed CA IX is now free to interact with the extracellular matrix within the tumor microenvironment without any constraints. In a parallel pathway, other exosomally associated cargo proteins such as galectins, which are known to be secreted by a non-canonical secretory pathway are also shed into the extracellular medium. These galectins may recognize specific ganglioside moieties on the exosomes and become associated with these vesicles. Apart from this, heat shock proteins and vascular endothelial growth factor are also known to be secreted by the non-classical pathway, possibly through exosomes. Together, the exosomally secreted CA IX as well as exosomally associated galectin, HSPs, VEGF and other proteins may participate in the modulation of the tumor microenvironment and modulate the protein-carbohydrate interactions in the extracellular matrix respectively, thus altering the intercellular communication processes, cause immunological escape, increased angiogenesis and increased activity of matrix metalloproteinas (MMPs) facilitating tumor progression. Further work is warranted to investigate this novel hypothesis.
Further studies are warranted to investigate the role of ganglioside shedding in relation to CA IX expression, function, tumor severity and immunological escape in renal cell carcinoma.

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Disclosure
The author reports no conflicts of interest.

References

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