

Review

HAVcR-1 involvement in cancer progression

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Summary. Formerly known for its importance in the immune system and kidney regeneration, it is becoming more apparent that HAVcR-1 is an important protein in cancer biology. HAVcR-1 is overexpressed in numerous cancers and associates with critical molecules of tight junctions. Tight junctions are critical in homeostasis of cellular compartments via the maintenance of epithelia and endothelia however it has also been suggested that via the prevention of dissemination, intravasation and extravasation, tight junctions play a critical role in the prevention of cancer metastasis. HAVcR-1 shedding and the production of the HAVcR-1 ectodomain has been linked to increased IL-6 thus implicated it in the process of angiogenesis via the activation of the STAT3 pathway leading to increased HIF-1 α . The HAVcR-1 ectodomain has also been shown to be a potential urinary biomarker in certain cancers. HAVcR-1 is potentially important molecule both for the detection of cancer and the treatment of cancer by being a novel target for anti-cancer therapeutics.

Key words: HAVcR-1, Tight junctions, Metastasis, Cancer, Ectodomain

Introduction

Cells are reliant on a wide array of transmembrane proteins to respond to external signals and coordinate cellular responses to these signals. The Hepatitis A virus cellular receptor (HAVcR-1), also termed T-cell Ig and

mucin domain containing molecule 1 (TIM-1) and kidney injury molecule 1 (KIM-1), is the cellular receptor for Hepatocellular Piconavirus the cause of acute hepatitis A in humans (Feigelstock et al., 1998). HAVcR-1 is a class I integral membrane glycoprotein, consisting of an N-terminal extracellular domain containing a cystine rich region, displaying homology to members of the immunoglobulin (Ig) superfamily, followed by an extended mucin like O-glycosylated threonine, serine and proline rich region, allowing accessibility to interactions with extracellular proteins (Kaplan et al., 1996).

As well as in hepatitis A, HAVcR-1 has been implicated in numerous other diseases. Although expressed on every tested human organ including: liver, small intestine, colon and spleen as well as high expression on the kidney and testis, the natural function of has not been fully investigated (Feigelstock et al., 1998). However it has been shown to be important in; the immune system whereby TIMs family of surface receptors are thought to regulate both physiologic and pathologic immune responses, in kidney repair whereby HAVcR-1 regulates survival and regeneration of cells and there is emerging evidence for HAVcR-1 importance in various cancers (Curtiss et al., 2011).

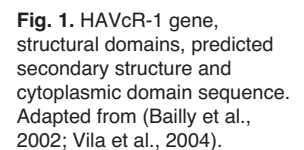
HAVcR-1 being overexpressed in numerous cancers has led to investigations into its role in cancer development (Vila et al., 2004). HAVcR-1 is involved in tight junctions (TJ), which have an imperative role in cell to cell adhesion to maintain distinct internal compartments. They are also thought to be important structure in cancer metastasis via the prevention of cancer cell dissemination as well as intravasation and extravasation. Thus the disruption of TJs has been linked to cancer metastasis and evidence suggests HAVcR-1 overexpression is responsible for this disruption, thus linking HAVcR-1 to cancer metastasis (Martin and

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Structure of HAVcR-1

2004).

The Cys-rich region, also known as the immunoglobulin (Ig) like domain, consisting of 109 amino acids, is the most homologous between simian and human HAVcr-1, containing six conserved cys residues and the first but not second N-glycosylation sites (Feigelstock et al., 1998, Bailly et al., 2002). The TSP rich region, also known as the mucin like domain, of 163 amino acids lies between the cys-rich region and TMD. Only 13 of the 27 consecutive repeats of the consensus PTTTTL remain in the human HAVcr-1 TSP rich region, but both N-glycosylation sites found in



A. Representation of the HAVcr-1 gene showing the size in base pairs of the introns number 1- 8 and the exons numbered in roman numerals 1-9.

B. Representation of HAVcR-1b protein showing the size and position of its structural domains, position of cysteine residues in the Ig-like domain represented by C, position of possible N-glycosylation sites represented by triangles, position of the tyrosine phosphorylation motif QAEDNIY represented by P and predicted cleavage site represented by a black box.

C. Predicted secondary structure of HAVcR-1.

D. Amino acid sequence of the cytoplasmic domain of HAVcR-1a and HAVcR-1b.

simian HAVcR-1 are conserved as well as a third possible N-glycosylation site located at residues 258 to 260 (Feigelstock et al., 1998). This TSP-rich region is predicted to be heavily glycosylated and to have an extended conformation, extending the cys-rich region away from the cell membrane like a stalk (Jentoft, 1990; Kuchroo et al., 2003). The intracellular domain of human HAVcR-1 is 48 amino acids, 12 amino acids shorter than that of the simian HAVcR-1, contains the tyrosine phosphorylation motif QAENIY (Fig. 1A) (Feigelstock et al., 1998; Bailly et al., 2002). Due to homology human HAVcR-1 is predicted to have a similar structure as simian HAVcR-1, known as the lollipop on a stick model (Fig. 1C) (Jentoft, 1990).

A second homologue was also cloned from rat KIM-1 termed HAVcR-1a (Ichimura et al., 1998). Thus the simian cloned HAVcR-1 is termed HAVcR-1b. HAVcR-1a was predicted to be 334 amino acids, showing 43.8% homology to rat KIM-1 with the Ig-like domain being the most homologous containing six conserved cysteines. It was also predicted that HAVcR-1a predominantly exists on the apical membrane of epithelial cells (Ichimura et al., 1998). HAVcR-1a is predominantly expressed in the liver whereas HAVcR-1b is predominantly expressed in the kidneys (Kuchroo et al., 2003). HAVcR-1a and b are identical except for the C-terminus, whereby HAVcR-1a is shorter than HAVcR-1b and therefore missing the tyrosine phosphorylation motif QAENIY (Fig. 1D) (Bailly et al., 2002). Genomic analysis and analysis of the cDNA product revealed that HAVcR-1a and b are splice variants (Bailly et al., 2002).

The complexity of HAVcR-1 leads to variability in protein size. The gene is expected to encode a 36kDa protein however due to four possible N-glycosylation sites, multiple possible O-glycosylation sites and possible biotinylation, it can result in the mature protein being approximately 100kDa as well as the immature protein being 70kDa or 50kDa. Due to proteolytic cleavage close to the TMD, a soluble ectodomain of HAVcR-1 is produced which is 10kDa less than the uncleaved protein, thus making it approximately 90kDa, whereas the membrane attached cleaved product is only 14kDa (Bailly et al., 2002).

Function in cancer

There have been numerous studies into the importance of HAVcR-1 in renal cell carcinoma (RCC). RCC is the most common type of kidney cancer in adults being responsible for approximately 80% of cases it is frequently diagnosed late making fatality rates high. The most common histological type of RCC, accounting for 75-80%, is clear cell RCC (ccRCC) (Cohen and McGovern, 2005). HAVcR-1 has been found to be overexpressed by 2-12 fold in 8/13 of ccRCC but interestingly expression is decreased in benign oncoyctomas (Vila et al., 2004). 60% of ccRCC contain duplications in chromosome 5 which has been mapped to Ch5q22-31.1 which contains the gene locus of

HAVcR-1, possibly explaining the increased expression of HAVcR-1 however transcriptional control, mRNA processing, mRNA export and protein stability may also contribute (Kaplan et al., 1996; Vila et al., 2004). Both the chromosomal location and overexpression of HAVcR-1 implicates it in the development of RCC and it is now thought that HAVcR-1 may be a susceptibility gene for RCC (Vila et al., 2004; Cuadros et al., 2013).

In 769-P and HK-2 kidney cell lines when differentiation is blocked and reverted with phorbol 12-myristate-13- acetate (PMA), HAVcR-1 expression is elevated. Furthermore overexpression of HAVcR-1 via transfection in 769-P cells resulted in the prevention of differentiation of the proximal tubule-derived cells and altered expression of other members of the family associated with differentiation/ de-differentiation events, suggesting HAVcR-1 is involved in the regulation of these events (Vila et al., 2004). HAVcR-1 has also been suggested to induce cell growth due overexpression and silencing in 769-P cells resulting in an increased or decreased proliferation index respectively (Cuadros et al., 2014). In wound healing experiments, overexpression of HAVcR-1 resulted in delayed migration and increased migration when expression was decreased (Cuadros et al., 2014). It may therefore be possible that the regulation of HAVcR-1 expression is important for multiple stages of cancer progression.

Ig-like domains are implicated in mediating protein-protein interactions at the cell surface especially cell-cell and cell-extracellular matrix (Kozarsky et al., 1988). The mucin domain which extends Ig-like domain away from surface like a stalk could have a role in configuration and protection as well as cell adhesion (Kozarsky et al., 1988; Jentoft, 1990). It is also possible that similarly to other cell surface mucins, such as MUC1, the mucin-like domain of HAVcR-1 may act in an anti-adhesive manner by preventing interactions between cells as well as between cells and the extracellular matrix (Komatsu et al., 1997; DeLoia et al., 1998). This may be a mechanism to allow detachment of cancer cells from primary tumours, a critical step in metastasis (Komatsu et al., 1997).

HAVcR-1 may therefore be a novel target for therapeutics in a variety of cancers and it has been shown that the monoclonal antibody 190/4 (mAb 190/4) binds HAVcR-1 and is internalized into the cell making it ideal for generation of an immunotoxin either by its conjugation with a toxin or its use in conjunction with a secondary antibody conjugated with a toxin (Weltman et al., 1987; Vila et al., 2004). The use of the mouse mAb 190/4 followed by a secondary anti-mouse antibody conjugated to the toxin saporin was shown to effectively kill the kidney cell line GL37 via the HAVcR-1 receptor, making it a possible anti-cancer treatment (Vila et al., 2004).

The HAVcR-1 ectodomain

Similarly to numerous integral plasma-membrane

proteins, HAVcR-1 has a soluble form released into the extracellular space produced by proteolytic cleavage close to the TMD and cells expressing HAVcR-1b are known to constitutively shed the ectodomain into the extracellular space (Hooper et al., 1997; Bailly et al., 2002). The cleavage of HAVcR-1 is predicted to be mediated by metalloproteases of the matrix metalloprotease (MMP) family or the a desintegrin and metalloprotease (ADAM) family due to cleavage being blocked by bitamastat (B4-94) and ilomastat (GM6001) which are broad band spectrum hydroxamic acid based zinc metalloprotease inhibitors. HAVcR-1 cleavage has also been shown to be increased by PMA treatment which is known to activate protein kinase C resulting in the shedding of many membrane proteins mediated by metalloproteases of the ADAM family. The cleavage site has been predicted to occur between residues 266 to 278 due to an antibody targeting the TSH-rich region blocking cleavage and due to shared homology in the extracellular domain both HAVcR-1a and b are equivalent substrates for proteases (Bailly et al., 2002; Zhang et al., 2007). HAVcR-1 shedding is enhanced by treatment with pervanadate, a protein tyrosine phosphatase inhibitor, which activates p38 and ERK-MAPK signaling. Inhibitors of p38 such as SB202190 blocked pervanadate induced shedding thus the ERK-MAPK signaling pathway appears to be important for HAVcR-1 shedding (Zhang et al., 2007).

Shedding of ectodomains has been reported in a variety of adhesion molecules. The ectodomains of these

molecules have been found to regulate cell adhesion either by being an antagonist for adhesion via down regulation or competition, or promoting adhesion via the ectodomain binding two cell surface and matrix components (Lochter et al., 1997; Kahn et al., 1998). Similarly to HAVcR-1, L1 is a type 1 membrane glycoprotein of the immunoglobulin superfamily. Proteolytic cleavage proximal to the plasma membrane produces soluble L1 which has been found to mediate cell migration by autocrine binding to $\alpha\beta 5$ integrins as well as integrin-mediated adhesion (Beer et al., 1999; Mechtersheimer et al., 2001). Although the effect of the HAVcR-1 ectodomain on adhesion and migration has not been fully investigated it has been shown that the Ig-like domain mediates the binding of soluble HAVcR-1 to a number of cell types.

The HAVcR-1 ectodomain has been shown to increase IL-6 expression which activates the STAT-3 pathway leading to increased HIF-1 α (Fig. 2) (Cuadros et al., 2014). High levels of IL-6 are present in patients with metastatic renal cell carcinoma (RCC) and are correlated with poor survival. IL-6 binds the ligand binding receptor gp80 which leads to the phosphorylation of tyrosine residues of the transducing receptor gp130. This allows for the docking and phosphorylation of the activator of transcription STAT-3 (Heinrich et al., 1998). STAT-3 transcriptionally activates genes involved in tumour proliferation, apoptosis inhibition and angiogenesis including HIF1 α , a key protein in promoting hypoxia induced

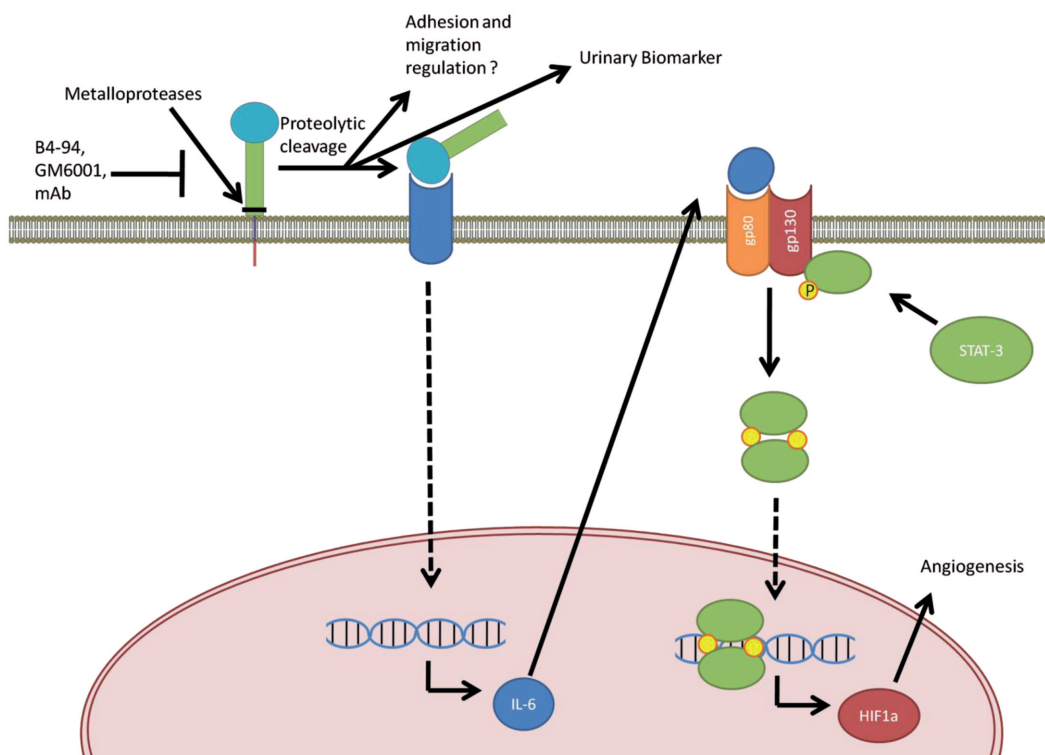


Fig. 2. The HAVcR-1 Ectodomain. Adapted from (Cuadros et al., 2014). Showing HAVcR-1 activation of the IL-6/ STAT-3 and potentially regulates angiogenesis as well as the other potential functions of the HAVcR-1 ectodomain.

angiogenesis (Jung et al., 2005).

HAVcR-1 shedding may therefore mediate angiogenesis and metastasis by regulating adhesion, migration and HIF-1 α levels thus could be targeted as therapeutic target. The production of soluble HAVcR-1 can be inhibited by small molecule inhibitors of metalloproteases. However similarly to Herceptin (Transtuzumab) blocking the proteolytic cleavage of HER2 in breast cancer, therapeutic monoclonal antibodies blocking the cleavage site of HAVcR-1 may be a more specific therapeutic in HAVcR-1 positive cancers (Molina et al., 2001; Bailly et al., 2002).

Mortality rates of cancer can in part be attributed to poor detection and screening methods. An example of this is in RCC, especially in the subtype clear cell RCC (ccRCC), where late presentation leads to high mortality rates, thus highlighting the need for improved screening techniques for this cancer. Radiological studies are not able to reliably distinguish between benign renal tumours and RCC and the use of such techniques for early screening is impractical and costly (Han et al., 2005; Millet et al., 2011). Percutaneous biopsies/fine needle aspiration used to distinguish histology has risks including; hemorrhage and tumour seeding along the needle track, another major drawback is the requirement of experienced cytopathologists which are not always available, all of which decrease the amount these procedures are performed (Lebret et al., 2007; Sahni et al., 2011).

Having previously been shown to be a biomarker of a wide variety of acute and chronic kidney diseases and the demand for a novel biomarker in RCC led to investigation into the potential use of urinary HAVcR-1 (Han et al., 2005; Morrissey et al., 2011). Formed via the shedding of HAVcR-1, urinary HAVcR-1 has the potential to distinguish between benign renal tumours and RCC as well as between ccRCC and other histological types of RCC (Han et al., 2005; Shalabi et al., 2013). There is also evidence that there is a correlation between the amount of urinary HAVcR-1 detected and RCC tumour size and grade (Han et al., 2005; Cuadros et al., 2013). The use of urinary HAVcR-1 may therefore lead to highly informative non-invasive testing. Low level of urinary HAVcR-1 have also been found in prostate cancer thus urinary HAVcR-1 has the potential to be a useful biomarker in other cancers as well as RCC (Han et al., 2005). In cancers where the HAVcR-1 ectodomain is not shed into the urine it may be shed into the circulation enabling HAVcR-1 to be a biomarker for other cancers.

HAVcR-1 in tight junctions

The maintenance of distinct fluid compartments is integral in multicellular organisms. These compartments are established by endothelial and epithelial sheets (Tsukita and Furuse, 1999). To enable the formation and maintenance of the distinct composition of adjacent tissue compartments the paracellular space between

epithelial/endothelial cells needs to be sealed to prevent non-specific solute diffusion (Anderson and Van Itallie, 1995). To allow transport of molecules between different compartments epithelial and endothelial cells must have different protein compositions on basolateral and apical membranes to allow for polarized transport (Tsukita and Furuse, 1999). To accomplish this tight junctions (TJ) seal the membranes of adjacent epithelial and endothelial cells leading to the obliteration of the intercellular space between these adjacent membranes thus acting as a barrier against paracellular transport as well as a fence to maintain apical and basolateral composition (Farquhar and Palade, 1963; Rindler et al., 1982; Giepmans and van Ijzendoorn, 2009).

TJ consist of three components; integral transmembrane proteins, peripheral/ plaque anchoring proteins and TJ associated/ regulatory proteins (Martin, 2014). Integral transmembrane proteins are essential adhesion proteins responsible for the correct assembly of TJ which is achieved by peripheral proteins, which act as a scaffold binding TJ molecules together and linking them to the actin cytoskeleton as well as signalling mechanisms, alongside associated/ regulatory proteins (Madara, 1987; Martin, 2014). Occludin, a 60kDa integral membrane protein was the first integral component of TJ to be identified (Furuse et al., 1993). The claudin family were the next components of TJ to be identified. Both claudin 1 and claudin 2 are 23kDa integral membrane proteins with 38% identical amino acid sequences to each other but no similarity to occludin amino acid sequence, however structurally they all contain 4 transmembrane domains (Furuse et al., 1998). Currently 27 claudin family members have been identified and alongside them and occludin other integral transmembrane proteins include: tricellulin, marvelD3 and junctional adhesion molecules (JAMs) (Mineta et al., 2011; Jiang et al., 2015). Numerous peripheral membrane proteins have also been identified including: zonula occludin-1 (ZO-1), ZO-2, ZO-3, cingulin, 7H6, Rab3B and AF-6 (Haskins et al., 1998). ZO-1, ZO-2 and ZO-3 are 225kDa, 160kDa and 130kDa respectively and belong to the MAGUK family (Stevenson et al., 1986; Jesaitis and Goodenough, 1994; Haskins et al., 1998). ZO-3 has been found to interact with ZO-1 and occludin and ZO-2 interacts with ZO-1 (Fig. 3) (Jesaitis and Goodenough, 1994; Haskins et al., 1998). TJ are therefore complex multiprotein structures and it is now becoming clear that they are not just responsible for cell polarity and blocking paracellular diffusion but also have roles in other cellular processes such as proliferation and differentiation as well as the prevention of metastasis (Latorre et al., 2005; Jiang et al., 2015). Over half of patients have already progressed to metastatic disease at time of diagnosis, thus tumour metastasis is the most life threatening event for cancer patients (DeVita et al., 1975).

The process of metastasis is complex, requiring numerous sequential events to successful, however it can be broken down into three main steps: invasion,

intravasation and extravasation. Invasion involves the loss of cell to cell contact thus allowing to dissociation of cancer cells leading to invasion of the surrounding stroma. Surrounding blood vessels formed by angiogenesis provide a route in which cancer cells can be transported to distant sites in the body. Thus the dissociated cancer cell must penetrate the endothelium to enter the circulatory system (intravasation) as well as to exit the circulatory system (extravasation) prior to forming secondary and tertiary foci (Martin, 2014). TJ exist between adjacent cancer cells in tumours of epithelia, thus act to prevent cancer cell dissociation, as well as existing between cells of endothelia, thus acting as a barrier against the movement of cancer cells through epithelia preventing intravasation and extravasation

(Martin et al., 2002; Martin and Jiang, 2009).

A number of virus receptors have been found to be associated with junctional structures including adherins and tight junctions. Investigations into the association of HAVcR-1 with junctional structures found via immunoprecipitation, the 50kDa HAVcR-1 associates with the C terminal of ZO-1 and to a lesser extent ZO-2 as well as the N-terminal of occludin and RhoC (Fig. 3) (Martin et al., 2011; Martin, 2014). Due to the importance of these molecules in tight junctions it is possible that HAVcR-1 is also involved in the tight junction complex in endothelial and epithelial cells. Overexpression and knockdown analysis of HAVcR-1 in HECV endothelial cells suggests the importance of HAVcR-1 expression in the hepatocellular growth factor

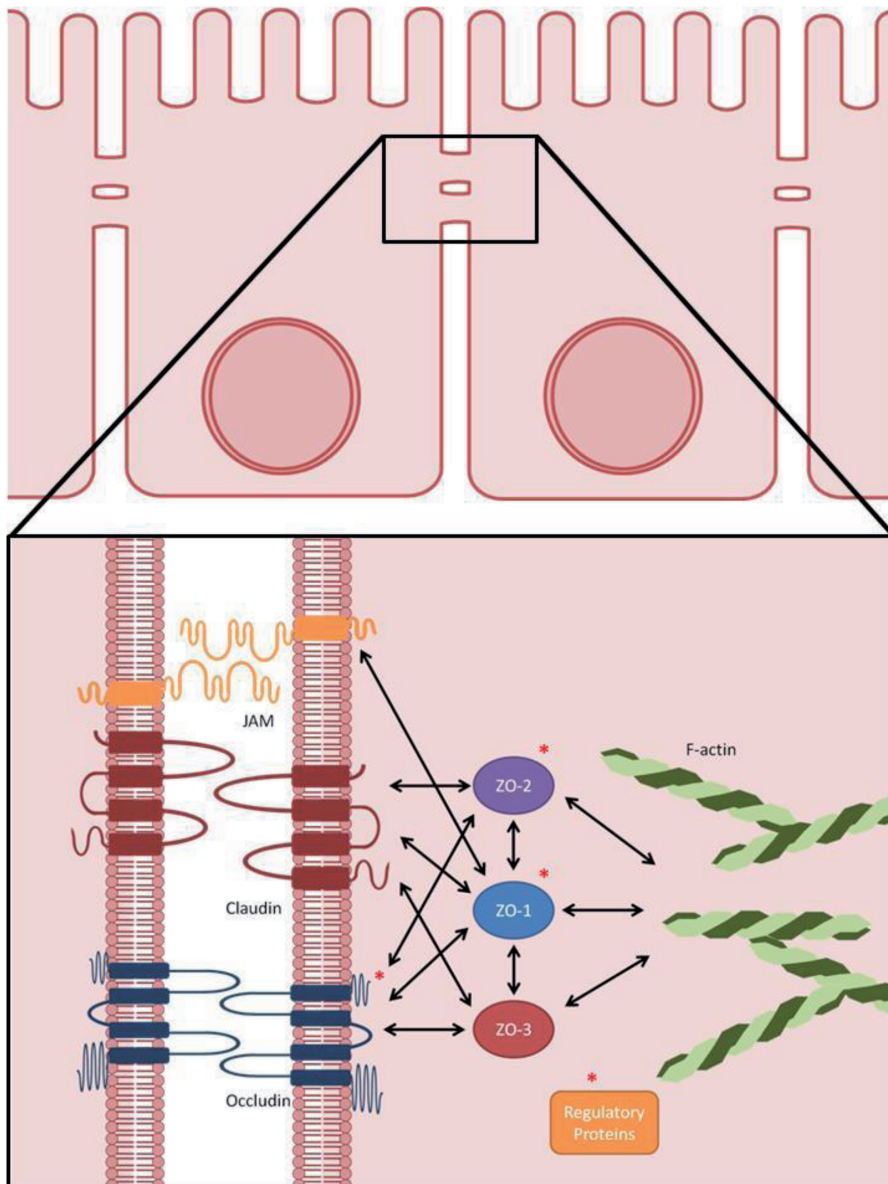


Fig. 3. Tight Junctions. Adapted from (Tsukita and Furuse, 1999; Shin et al., 2006). Representation of epithelial cells connected via tight junctions with magnified representation of molecular components of tight junctions shown and component found to interact with HAVcR-1 indicated by red stars.

(HGF) mediated breakdown of TJ. This was shown by decreased transepithelial resistance in HAVcR-1 overexpressed HECV cells in comparison to HAVcR-1 knockdown HECV cells when treated with HGF. Dual immunofluorescence of HAVcR-1 and ZO-1 showed an increased expression and concentrated disruption of ZO-1 in cell-cell junctions in knockdown HECV cells in comparison to wild type HECV cells when treated with HGF. Therefore it appears likely that both HGF and HAVcR-1 act on the same pathway responsible for the integrity and maintenance of TJ (Martin, 2014). Overexpression of HAVcR-1 in cells results in decreased TJ, therefore HAVcR-1 overexpression in cancer is likely to also result in decreased TJ which may mediate metastasis. HAVcR-1 may therefore be a target for anti-metastatic cancer therapies.

Conclusions and perspectives

HAVcR-1 is a type I transmembrane glycoprotein which exists in two splice variants known as HAVcR-1a and HAVcR-1b consisting of 334 and 359 amino acids respectively. The variation in size is due to HAVcR-1a having a smaller cytoplasmic domain than HAVcR-1b, the effect of which has not been investigated. Due to homology in the extracellular domain both variant are subject to metalloprotease cleaving releasing an ectodomain, the amount of which correlates with the amount of IL-6 expression. This therefore links HAVcR-1 to angiogenesis via the IL-6/STAT-3/HIF-1 α pathway. HAVcR-1 has also been linked to metastasis via the regulation of TJ preventing cancer cell dissemination, intravasation and extravasation. Both processes are critical for cancer development thus making HAVcR-1 a potential target for cancer therapeutics however further investigation to determine the functions of HAVcR-1 is required. The HAVcR-1 ectodomain being present in the urine of certain cancer makes it a potential biomarker however the accuracy of its use needs to be determined as well as investigations into the use of HAVcR-1 as a biomarker in other cancers.

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