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TMPRSS4: an emerging potential therapeutic target in cancer

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Altered expression and activity of proteases is a key event in cancer, particularly in relation to invasion, modification of the extracellular matrix and metastasis. The transmembrane protease, serine 4 (TMPRSS4) is closely related to other cancer-associated proteases, such as hepsin, TMPRSS2 and matriptase. We review in this study up-to-date information about expression, role, regulation and clinical relevance of TMPRSS4 in cancer. Increased expression of this protease is associated with acquisition of epithelial to mesenchymal transition, invasion and metastasis *in vivo*. Signalling in cancer cells involves upregulation of integrin-α5 (ITG-α5) and urokinase-type plasminogen activator (uPA), downregulation of E-cadherin and activation of uPA enzymatic activity at the plasma membrane, as well as phosphorylation of FAK, Src, Akt and ERK1/2 intracellularly. Upregulation of miR-205 hinders the protumorigenic effects elicited by TMPRSS4 through restoration of E-cadherin levels and direct targeting of ITG-α5. High levels of TMPRSS4 have been found in several types of solid tumours in patients, and association with poor prognosis has been consistently described. On the basis of this information and the structural characteristics of this druggable protease, we suggest that TMPRSS4 could be a novel potential therapeutic target in solid tumours.

TMPRSS4, A TRANSMEMBRANE SERINE PROTEASE

Proteolysis is a regulatory mechanism mediated by specific hydrolysis of peptide bonds (Lopez-Otin and Overall, 2002). Due to this post-translational modification, the role of numerous proteins is controlled by proteases, which then modulate a plethora of cellular mechanisms, such as cell growth, apoptosis, protein secretion, phagocytosis, signal transduction and extracellular matrix turnover (Puente *et al*, 2005). In humans, more than 2% of the genes code for a complex system of more than 700 proteases and inhibitors of proteases. The dysregulation of protease activity is related to different pathologies, including arthritis, cancer and neurogenerative and cardiovascular diseases (Puente *et al*, 2005).

On the basis of their mechanisms of catalysis, proteases are classified into serine, aspartyl, metallo, threonine and cysteine proteases (Puente *et al*, 2005). This large family of proteins can be found extracellularly, at the cellular surface, in the cytoplasm or within specific subcellular structures such as lysosomes. Some serine proteases exhibit a transmembrane domain through which they get anchored to the plasma membrane. Cell surface proteolysis has emerged as an important mechanism for the generation of biologically active factors that mediate a diverse range of cellular functions. Depending on the structure of the transmembrane domain, these serine proteases can be classified into three groups: type I (with a carboxy-terminal transmembrane domain), type II or TTSP (with an amino-terminal transmembrane domain spanning through the cytosol) and GPI (bound to the membrane by glycosyl-phosphatidylinositol; Netzel-Arnett *et al*, 2003). The type II family of serine proteases includes 20 members that are subdivided into four subfamilies: matriptase, hepsin/transmembrane protease/serine (TMPRSS), HAT/differentially expressed in squamous cell carcinoma (DESC) and corin (Szabo and Bugge, 2008). In this review, we summarise the state of the art about the expression, role, signalling and clinical relevance of TMPRSS4 in cancer, where the importance of this membrane-bound serine protease is beginning to be acknowledged.

TMPRSS4 STRUCTURE AND CATALYTIC ACTIVITY

The transmembrane protease, serine 4 (TMPRSS4), previously referred to as TMPRSS3 (Wallrapp *et al*, 2000), is localised in the

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long arm of chromosome 11 (11q23.3). This gene contains 48,597 bp and consists of 13 exons and 12 introns. Eighteen different transcripts of this gene can be generated, 2 of which are degraded due to nonsense-mediated decay and 8 of which do not give rise to a protein product. Of the remaining eight, three have incomplete coding sequence (CDS) in 5′ or 3′ and do not have either start or stop codon sequences. Accordingly, the TMPRSS4 gene codes for five isoforms with complete CDS. The canonic protein (TMPRSS4-1) is composed of 437 amino acids (with a predictive size of 48 kDa and two glycosylation sites at 130 and 178 amino acids), whereas isoforms 2 and 3 differ in two and five amino acids, respectively (http://www.ensembl.org/index.html).

TMPRSS4 shares the following domains with the other TTSP family members: proteolytic, stem, transmembrane and cytoplasmic domains (Figure 1; Hooper *et al*, 2001). The proteolytic domain is highly conserved between different TTSPs and its activity is dependent on the presence of a 'catalytic triad' that includes the amino acids His, Asp and Ser. Enzymatic activity is also modulated by a substrate-binding pocket that determines the enzyme's specificity (Antalis *et al*, 2010). The stem region may include different regulatory and/or binding domains, as is the case for one LDL receptor class A domain present in TMPRSS4. In other TTSPs, this domain binds Ca^{2+} ions and has a role in the internalisation of macromolecules (Daly *et al*, 1995). The scavenger receptor domain is also present in the stem region. This domain is involved in binding of lipoproteins, lipids and polysaccharides (Netzel-Arnett *et al*, 2003). The remaining domains consist of the transmembrane region and a short cytoplasmic tail whose putative biological function in terms of cell signalling or cytoskeletal attachment is at present unknown (Hooper *et al*, 2001; Netzel-Arnett *et al*, 2003).

It is thought that all TTSPs are synthesised as zymogens that would need to be activated by proteolytic cleavage in a highly conserved motif that precedes the catalytic domain. On cleavage, it is likely that a disulphide bond maintains the catalytic domain linked to the rest of the protein and, therefore, anchored to the membrane (Hooper *et al*, 2001; Netzel-Arnett *et al*, 2003). Many TTSPs have been shown to be activated by autocatalysis, including TMPRSS2, matriptase, hepsin and TMPRSS4 (Szabo and Bugge, 2008). Other proteases can also activate pro-TTSP zymogens; activation by enterokinase has been suggested for TMPRSS4 (Min *et al*, 2014). Nonetheless, current knowledge about TMPRSS4 processing needs to be better substantiated.

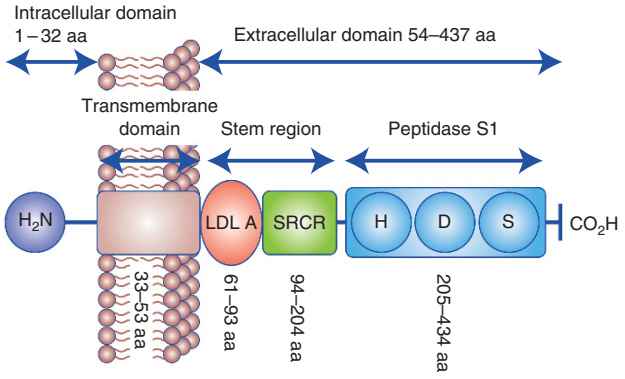

Figure 1. Schematic diagram of the TMPRSS4 structure. TMPRSS4 is a single-pass type II membrane protein. It contains a serine protease domain at the C terminus (peptidase S1), followed by a scavenger receptor cysteine-rich domain (SRDR) and a low-density lipoprotein receptor class A domain. H, D and S in the serine protease domain indicate the position of the three catalytic residues histidine, aspartate and serine, respectively.

The existence of soluble forms of TTSPs has been described for HAT, enteropeptidase, matriptase and TMPRSS13 (MSPS), which implies that fragments of these proteases could be secreted (Hooper *et al*, 2001; Kim *et al*, 2001). The active TMPRSS4 protease domain can be released from cells in culture and are found in the conditioned medium (Min *et al*, 2014). This interesting finding opens the possibility that soluble fragments could be detected in serum of tumour-bearing patients and, therefore, that TMPRSS4 can be used as a non-invasive diagnostic marker.

Although the specific substrates of TMPRSS4 are still under-explored, three proteins have been identified so far: (a) hemagglutinin of the influenza virus, which is necessary for virus infection (Bertram *et al*, 2010); (b) the urokinase-type plasminogen activator (uPA), whose activation enhances cancer cell invasion (see below in the next section); and (c) the epithelial sodium channel, on cleavage of the γ subunit (Passero *et al*, 2012).

ROLE OF TMPRSS4 AND ACTIVATION OF INTRACELLULAR PATHWAYS IN CANCER

Among other proteases with clinical potential in cancer, such as uPA, matriptase, furin or stromelysin, TMPRSS4 could emerge as a new potential candidate. TMPRSS4 has been involved mainly in two functions at present: embryo development and cancer. In zebrafish embryos, this protease is necessary for organogenesis, as TMPRSS4 knockdown using morpholinos resulted in severe defects in tissue development and cell differentiation, including a disturbed skeletal muscle formation, a decelerated heartbeat and a degenerated vascular system (Ohler and Becker-Pauly, 2011). This result suggests that TMPRSS4 may modulate the activity of adhesion molecules involved in organ development (Ohler and Becker-Pauly, 2011). Generation of knockout and transgenic mice (lacking at this moment) would allow studying the involvement of this protease in healthy and pathological conditions, in a more relevant way for human diseases.

Most data about TMPRSS4 come from cancer development and metastasis studies. Its overexpression in tumours has been reported in pancreatic (Wallrapp *et al*, 2000), ovarian (Takahashi *et al*, 2013), thyroid (Ohler and Becker-Pauly, 2011), colorectal (Jung *et al*, 2008; Kim *et al*, 2010), lung (Larzabal *et al*, 2011; Figure 2), breast (Cheng *et al*, 2013b; Liang *et al*, 2013), cervical (Cheng *et al*, 2013b), gallbladder (Wu *et al*, 2014), gastric (Luo *et al*, 2013; Sheng *et al*, 2014) and liver cancer (Li *et al*, 2011).

Whether increased expression in cancer may be due to gene amplification, chromosome rearrangements, transcriptional

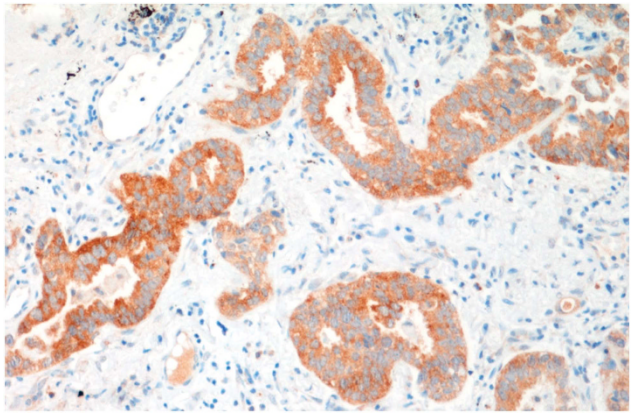

Figure 2. Immunohistochemical staining of a lung cancer specimen to localize TMPRSS4.

dysregulation or other mechanisms is still unknown. In normal liver, TMPRSS4 and TMPRSS13 promoters have been shown to be methylated, whereas in hepatocellular carcinoma these genes are hypomethylated (Stefanska *et al*, 2011). These data suggest that a possible epigenetic dysregulation could be partially responsible for the increased expression observed in cancer. Nonetheless, these preliminary findings need further validation and expansion to other tumour types. TMPRSS4 has been shown to increase in hepatocarcinoma cells 30 days after irradiation, when expression of a first wave of VEGF- and MMP-9-induced cell response genes returns to normal levels (Li *et al*, 2011). Overexpression of TMPRSS4 in the second wave of long-term response was critical for cell dissemination and metastasis of these cells (Li *et al*, 2011).

Several colon, lung and breast cancer cell lines express TMPRSS4 and have been used as *in vitro* models to uncover the role and signalling of TMPRSS4 (Jung *et al*, 2008; Larzabal *et al*, 2011). Migration and invasion are hallmarks of TMPRSS4 function in cancer cells. In lung and colon cancer, inhibition of this protease reduces migration (Jung *et al*, 2008; Larzabal *et al*, 2011) and invasion through matrigel, collagen type I and fibronectin-1 (Jung *et al*, 2008). Conversely, TMPRSS4 overexpression enhances migration and invasion in colon cancer (Jung *et al*, 2008). We have also reported an inhibition in the proliferation rates of lung cancer cell lines transfected with TMPRSS4-specific shRNA (Larzabal *et al*, 2011).

TMPRSS4 is responsible for the acquisition of an epithelial to mesenchymal transition (EMT) phenotype as well (Jung *et al*, 2008; Larzabal *et al*, 2011). In colon cancer cells, its overexpression leads to an intracellular signalling cascade that involves FAK, ERK1/2, Akt, Src and Rac1 activation (Kim *et al*, 2010; Figure 3). FAK and Rac1 signalling (which induces lapellipodia formation) are required for TMPRSS4-mediated invasion, changes in cell morphology and EMT (Kim *et al*, 2010). This pathway activates the transcription factors SIP1/ZEB2 and promotes E-cadherin loss (Jung *et al*, 2008; Li *et al*, 2011), a key event involved in EMT (Kim *et al*, 2010; Larzabal *et al*, 2011). In TMPRSS4-overexpressing cells, inhibition of PI3K or Src with specific compounds reduces invasiveness and causes actin reorganisation without restoration of E-cadherin expression. In addition, TMPRSS4 upregulates integrin-α5 (ITG-α5) to induce invasiveness (Kim *et al*, 2010).

To identify new molecular mechanisms elicited by TMPRSS4, our group conducted transcriptomic profiling of TMPRSS4 knocked down lung cancer cells. MIR205HG, the gene coding for miR-205, a micro-RNA that suppresses metastasis (Iorio *et al*, 2009), was found to be overexpressed on TMPRSS4 down-regulation (Larzabal *et al*, 2014). Increased levels of miR-205 impaired significantly cell growth (causing a G_0/G_1 cell cycle arrest), migration and attachment to fibronectin-1, and produced tumour shrinkage; moreover, we demonstrated that ITG-α5 is a new direct target of miR-205 in NSCLC and proposed a novel regulatory pathway involving TMPRSS4/miR-205/ITG-α5 (Larzabal *et al*, 2014). Therefore, data from both colon and lung cancer studies suggest a close relationship between TMPRSS4 and ITG-α5. Of note, blocking ITG-α5 antibodies (volociximab) are being evaluated in clinical trials for cancer treatment. The possibility of co-targeting both proteins could be an interesting approach to assess synergistic anti-tumour efficacy.

TMPRSS4 regulates uPA by a dual mechanism through increased gene expression and processing of pro-uPA into its active form (Min *et al*, 2014), which leads to enhanced invasion. uPA overexpression was mediated by JNK and transcription factors Sp1, Sp3 and AP-1. Moreover, immunohistochemical studies have shown co-expression of both proteases in human lung and prostate cancer tissues (Min *et al*, 2014). Therefore, it is possible that TMPRSS4 and uPA cooperate in tumours to accelerate metastasis.

Because of all these protumorigenic effects, TMPRSS4 has been suggested as a potential therapeutic target. Screening of a library of compounds against TMPRSS4 serine proteas activity identified several classes of inhibitory compounds, in particular a novel series of 2-hydroxydiarylamide derivatives (Kang *et al*, 2013). The leader compounds exhibited a relatively decent IC_{50} (6–12 μM), but invasion assays in matrigel using TMPRSS4-overexpressing SW480 cells revealed a modest inhibitory effect. Therefore, new families of more effective compounds should be developed and tested in different *in vitro* and *in vivo* models to support the translation of anti-TMPRSS4 therapy in clinical settings.

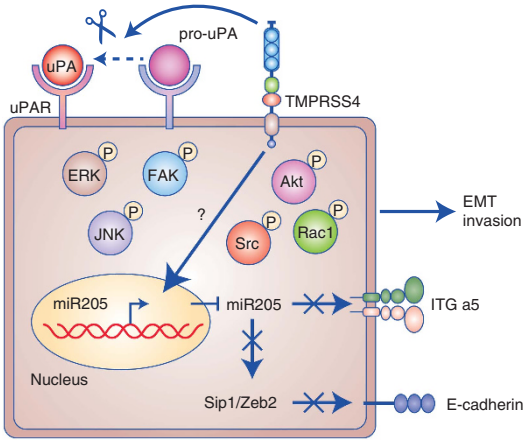

Figure 3. Scheme of molecules that participate in TMPRSS4-mediated signaling. Intracellular mediators include phosphorylated ERK, JNK, Akt, Src, FAK and Rac1. miR-205 targets integrin-α5 (ITG-a5), which is responsible for TMPRSS4-mediated invasiveness and EMT. miR-205 also targets Sip1/Zeb2, a repressor of E-cadherin. At the plasma membrane, TMPRSS4 cleaves the inactive form of uPA (pro-uPA) to accelerate invasiveness.

PROGNOSTIC VALUE OF TMPRSS4

Preclinical data showing increased malignancy in cells with high TMPRSS4 expression are in keeping with studies in human subjects. As mentioned in the previous section, TMPRSS4 is overexpressed in many solid tumours; importantly, this expression has been associated with poor outcome. In colorectal cancer, high TMPRSS4 protein levels were significantly correlated with advanced TNM stage and predicted shorter overall survival (OS) and disease-free survival (DFS). In Cox regression analysis, TMPRSS4 was an independent predictive factor of both OS and DFS (Huang *et al*, 2013). Similar results have been reported in patients with salivary adenoid cystic carcinoma (Dai *et al*, 2013). Analysis by western blot revealed a >30-fold increase in TMPRSS4 levels in these tumour tissues compared with matched non-cancerous tissues. In this study, TMPRSS4 levels also correlated with TNM stage, as well as lymph node and distant metastasis. Multivariate analysis revealed that TMPRSS4 was an independent predictor of both OS and DFS.

In breast cancer, two independent studies have demonstrated the prognostic value of TMPRSS4 (Cheng *et al*, 2013a; Liang *et al*, 2013). In a series of 109 patients, protein levels of TMPRSS4 in tumours were significantly higher than those of non-malignant tissues. High expression of this protease correlated with lymph

node metastasis, histopathological grade and tumour size (>2 cm), but not with oestrogen, progesterone or HER2 receptors. In univariate and multivariate analysis, high TMPRSS4 levels were associated with both DFS and OS (Liang *et al*, 2013). The other study found that high TMPRSS4 levels were observed in 62.4% breast cancer tissues (from a total number of 181 patients). The frequency of tumours with high TMPRSS4 expression was significantly higher in triple-negative breast cancer patients (73.2%) than in non-triple-negative patients. They also confirmed correlation between high TMPRSS4 expression and lymph node metastasis and tumour size. Association between TMPRSS4 and reduced DFS and OS was found for both triple-negative and non-triple-negative tumours.

In keeping with *in vitro* studies that evidenced activation of ERK1/2 as a result of TMPRSS4 signalling, expression of both proteins in clinical samples from gastric cancer patients ($n=436$) was found to be statistically correlated (Luo *et al*, 2013). High levels of TMPRSS4 were also associated with tumour size, lymph node and distal metastases, and TNM stage. Further multivariate analysis in these patients demonstrated that expression of TMPRSS4 was an independent prognostic factor. In a different study, TMPRSS4 was confirmed as an indicator of poor prognosis in gastric cancer (Sheng *et al*, 2014). The prognostic value of TMPRSS4 has also been recently shown in gallbladder cancer as well (Wu *et al*, 2014).

CONCLUSION

Increasing data on the role of TMPRSS4 in cancer development and metastasis suggest that this membrane-anchored serine protease merits further consideration as a novel potential therapeutic target in solid tumours. Although data in the literature on TMPRSS4 are still scarce, several lines of evidence support this statement: (a) TMPRSS4 has been proved to promote metastasis in preclinical models; (b) overexpression has been found in a variety of cancer types compared with normal tissues; (c) high levels are consistently associated with reduced DFS and OS; (d) it is a membrane-bound protein, which makes it an attractive target for the development of blocking antibodies or other biological inhibitory tools; and (e) TMPRSS4 extracellular fragments are shed into conditioned media of cultured cells, suggesting that they could be potentially found in serum samples from patients. We conclude that TMPRSS4 is an emerging potential cancer target, although future studies should determine important mechanistic and translational aspects of this protease before being considered as a consolidated target.

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