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From Cell Biology to Tissue Engineering

Development of biological tools to assess the role of TMPRSS4 and identification of novel tumor types with high expression of this prometastatic protein

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Summary. Metastatic spread is responsible for the majority of cancer deaths and identification of metastasis-related therapeutic targets is compulsory. TMPRSS4 is a pro-metastatic druggable transmembrane type II serine protease whose expression has been associated with the development of several cancer types and poor prognosis. To study the role and expression of this protease in cancer, we have developed molecular tools (active recombinant proteins and a polyclonal antibody) that can be used for diagnostic purposes and for testing anti-TMPRSS4 drugs. In addition, we have evaluated TMPRSS4 protein expression in several cancer tissue microarrays (TMAs). Full length and truncated TMPRSS4 recombinant proteins maintained the catalytic activity in two different expression systems (baculovirus and *E. coli*). Sensitivity of the rabbit polyclonal antisera against TMPRSS4 (ING-pAb) outperformed the antibody most commonly used in clinical settings. Analysis by immunohistochemistry in the different TMAs identified a subset of adenocarcinomas, squamous carcinomas, large cell carcinomas and carcinoids of the lung, in which TMPRSS4 expression may define aggressive tumors. In conclusion, our biological tools will help the characterization of TMPRSS4 activity and protein expression, as well as the evaluation of anti-TMPRSS4 drugs. Future studies should determine the clinical value of assessing TMPRSS4 levels in different types of lung cancer.

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Introduction

Cancer remains a serious epidemiological problem worldwide. According to the International Agency for Research on Cancer (IARC), there were 14.1 million new cancer cases and 8.2 million cancer deaths in 2012 worldwide (Ferlay et al., 2015). Based on the EUCAN estimates, more than 3.45 million new cancer cases were diagnosed in Europe. The most common cancer types are breast, colorectal, prostate and lung, which represent half of the overall burden of cancer in Europe (Steliarova-Foucher et al., 2015).

Metastasis, the main cause of cancer-related death in patients, is a multistep process that involves evasion of cancer cells from the primary tumor, spread through blood or lymphatic vessels, and the colonization of new organs. Proteases play a key role in metastasis, since malignant cells need to digest the extracellular matrix to migrate and establish a metastatic niche (Antalis et al., 2010; Ohler and Becker-Pauly, 2012). In addition, proteases activate growth factors that reside in the extracellular matrix in an inactive form. Because of these key functions, deregulation of proteases constitutes one of the hallmarks of cancer development (Hanahan and Weinberg, 2011; López-Otín and Hunter, 2010).

The S1 class of serine proteases (type II trans-membrane serine proteases, TTSPs) was described in 2003 (Netzel-Arnett et al., 2016). Many of these

proteases have been found to be overexpressed in a variety of tumors, which suggests their potential role in cancer growth (Antalis et al., 2010). These types of proteases are anchored to the plasma membrane and their localization in the cell surface allows them to mediate signal transduction between the extracellular environment and the cell (Antalis et al., 2010). Among the TTSPs, TMPRSS4 has emerged as a putative target in some solid tumors (de Aberasturi and Calvo, 2015; Ohler and Becker-Pauly, 2012). TMPRSS4 is a single-pass transmembrane protein that contains 437 amino acids with a short intracellular domain and a large external part with scavenger receptor Cys-rich (SRCR), low density lipoprotein (LDL) receptor and proteolytic domains (Ohler and Becker-Pauly, 2012; de Aberasturi and Calvo, 2015). TMPRSS4 proteolyzes and activates the haemagglutinin of the influenza virus to allow viral infection, the ENAc plasma membrane channel (Passero et al., 2012) and the pro-urokinase-type plasminogen activator (pro-uPA), a well-known protein involved in invasion and metastasis (Min et al., 2014; de Aberasturi and Calvo, 2015).

Experimental studies have found that TMPRSS4 induces epithelial to mesenchymal transition (EMT) and, as a consequence, enhances metastasis (Jung et al., 2008; Kim et al., 2010). Induction of the EMT phenotype by TMPRSS4 is mediated by upregulation of integrin-$\alpha5\beta1$ and activation of downstream signalling cascades involving phosphorylation of kinases (Kim and Lee, 2014; Larzabal et al., 2014; Wang et al., 2015). We have previously demonstrated that integrin $\alpha5\beta1$ is a novel direct target of miR-205, a micro-RNA regulated by TMPRSS4 (Larzabal et al., 2014). Lee et al. have recently shown that TMPRSS4 enhances proliferation and invasion through increased expression of Slug and cyclin-D (Lee et al., 2016). Our recent work has also shown that TMPRSS4 induces cancer stem cell properties in lung cancer cells and that its expression correlates with ALDH and OCT4 levels in NSCLC patients (de Aberasturi et al., 2016). These data suggest that TMPRSS4 expression in cancer cells is associated with a malignant phenotype. Knock-down of TMPRSS4 in zebrafish showed an impairment of cell adhesion and organogenesis (Ohler and Becker-Pauly, 2011). However, no evident defects were reported in mice lacking this gene (Keppner et al., 2015).

In clinical samples, TMPRSS4 has been described to be upregulated and associated with poor prognosis and/or tumor stage in high incidence cancer types, such as colorectal (Kim et al., 2010), breast (Cheng and Hong Kong, 2013a,b), liver (Wang et al., 2015), cervical (Cheng and Hong Kong, 2013a,b) and lung (Larzabal et al., 2014; Villalba et al., 2016). Therefore, this protease might represent a novel prognostic marker. In spite of the fact that several commercial polyclonal antibodies have been developed against TMPRSS4, in our hands, some of them are not reliable (unpublished observations). Since the prognostic value of TMPRSS4 is being increasingly studied, this is essential to define the clinical value of the expression of this protease. In addition, testing novel drugs or biological therapies targeting TMPRSS4 needs the development of biochemical systems where the protein activity can be screened and tested. Therefore, the goals of the present study were as follows: To develop recombinant active TMPRSS4 (full length and proteolytic-containing fragments) produced in different expression systems; to develop a polyclonal antibody that can be used in immunohistochemical studies; and finally, to test expression of TMPRSS4 in a panel of malignant tissues with the aim of identifying novel cancer types where this protease may constitute a biomarker for future clinical studies assessing prognosis.

Materials and methods

Cells, bacterial strains and viruses to generate TMPRSS4 recombinant proteins

Spodoptera frugiperda clone 9 cell line (Sf9f, CRL 1711, ATCC) was used to propagate recombinant baculoviruses. Cells were grown at 27°C using Grace's insect tissue culture medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) (Gibco), 0.2% Pluronic F-68 (Sigma-Aldrich, Steinheim, Germany), and antibiotics. *Escherichia coli* strain BL21-AI One Shot was used for plasmid transformation.

Recombinant baculoviruses AcHLTA-TMPRSS4, expressing either the complete form or fragments of the TMPRSS4 type II transmembrane serine protease, were used for protein preparation as previously described (Rueda et al., 1999a,b).

Cloning of TMPRSS4 gene using Gateway System (GW)

The pDONR223 plasmid containing the full-length human TMPRSS4 gene (1-437 aa protein) was purchased from Open Biosystem BC014245 and subcloned into the pDEST17-GW *E. coli* expression vector and pAcHLTA-GW baculovirus expression vector, which was previously designed in the laboratory using the Gateway® System (GW, Invitrogen, Carlsbad, CA). Both vectors were used to construct the recombinant proteins with a polyhistidine tag in the amino terminus, to facilitate its monitoring during the expression and purification processes. Truncated mutants containing either the whole extracellular domain or the catalytic domain (Fig. 1) were generated by PCR amplification using specific oligonucleotides from the pDONR223 cDNA (Table 1). To improve the efficiency of the protease activity of the short recombinant protein containing the catalytic domain, an ATGGGA sequence was added in the forward primer after the ATG initiation codon, as previously recommended (Qiu et al., 2009) (Table 1). For cloning purposes of the truncated proteins, a stop codon sequence was added in front of the COOH-terminus. The amplified DNA fragments were cloned into the expression vectors mentioned before. All

sequences were confirmed with an ABI PRISM 3130 DNA automatic sequencer.

Expression, purification and characterization of TMPRSS4 proteins from E. coli

The recombinant clones pDEST-TMPRSS4 (1-437 aa), pDEST-TMPRSS4 (54-434 aa), and pDEST-TMPRSS4 (205-434 aa) were grown at 37°C in LB containing 100 μg/ml ampicillin. When the culture reached an OD600 of 0.4-0.6, protein expression was induced by adding 0.2% L- arabinose, and cultures were grown at 37°C for 3h. Bacterial cells were collected by centrifugation at 6.000xg for 5 min at 4°C. Recombinant proteins were purified by solubilization procedures using the following buffer: 50 mM Tris–HCl, 50 mM NaCl, 0.5 mM EDTA, 5 mM TCEP, 5% glycerol and 30% N-Lauroylsarcosine (pH 8). Refolding of the purified proteins was achieved by sequential dialysis with 10 mM Tris–HCl, 0.05 mM EDTA, 0.1 mM TCEP, and 0.06% (w/v) N-Lauroylsarcosine (pH 8). Purified proteins were stored at -20°C.

Protein concentration was determined by densitometric assays and then analysed by SDS-PAGE and transferred to a nitrocellulose membrane by semi-dry method using Trans-Blot SD Electroforetic Transfer Cell (BIO-RAD). The commercial anti-His monoclonal antibody (GE Healthcare, UK) and the specific rabbit antiserum against TMPRSS4 produced at INGENASA (ING-pAb), were used as primary antibodies. After 1h incubation with the primary antibody at room temperature (RT), the membrane was exposed to anti-mouse or anti-rabbit secondary antibodies conjugated with horseradish peroxidase at 1:2000 dilution for 1h. The immune complexes were detected by addition of tetramethylbenzidine (TMBMAX, Neogen Corporation) or using the Lumi Light Plus chemiluminiscence kit (Roche, Mannheim, Germany).

Expression and purification of TMPRSS4 proteins in baculovirus system

This expression system was used to generate recombinant TMPRSS4 (1-437 aa) and the truncated mutant TMPRSS4 (205-434 aa) proteins. Sf9 cells were transfected with a mixture of the baculovirus transfer vector (2 μg) and linearized BacPak6 DNA (500 ng) in the presence of Jet PEI™ . After 5 to 6 days, the culture supernatant was plated for baculovirus isolation in the presence of X-Gal^{n} (5-bromo-4-chloro-3-indolyl-B-Dgalactopyranoside), white plaques were recovered, and the recombinant baculoviruses were purified, as

Table 1. Primer sequences. The initiation and stop codons are shown in bold.

Primers	Sequence	Position (nt)
TMPRSS4-N160 (FW)	**ATG**AAGGTGATTCTGGATAAATAC	160-180
TMPRSS4-N613 (FW)	**ATG**ATGGGAGTGTGGTGGGTGGG	613-624
TMPRSS4-C1283 (REV)	**TTA**CTTCCAGACATTGTAGATCCA	1302-1283

FW: Forward primers; REV: reverse primers.

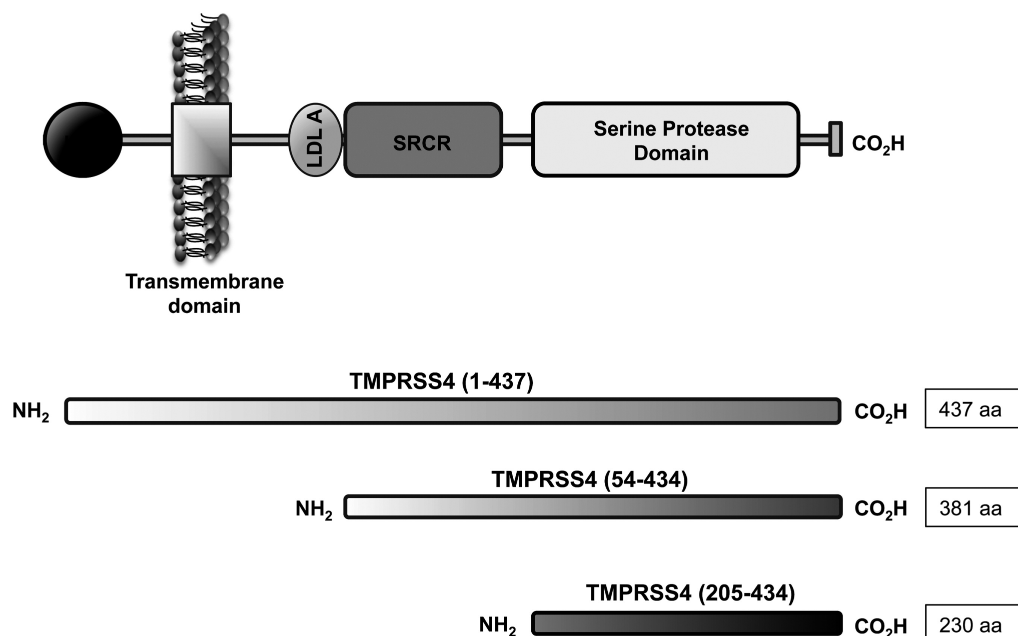

Fig. 1. Scheme of the TMPRSS4 native protein showing its different domains, and the recombinant proteins produced in *E. coli* or baculovirus systems. The acronyms for the TMPRSS4 domains are as follows: LDLA: low-density lipoprotein receptor class A domain; SRCR: scavenger receptor cysteine-rich domain. The serine protease catalytic domain is located at the C terminus (peptidase S1), in the extracellular part of the cell.

previously described (Rueda et al., 1999a,b). Sf9 cells ($1x10^6$ cells/ml) were infected at a multiplicity of infection of 3 plaque-forming units (pfu) per cell, following published protocols (Rueda et al., 1999a,b). Cells were collected 72h postinfection. The method of purification and refolding was the same described above for *E. coli*. The identity of the purified TMPRSS4 recombinant protein was determined by SDS-PAGE and Western blot, as previously explained.

Serine protease activity assay

The study of serine protease activity of the TMPRSS4 recombinant proteins was carried out after the refolding process with the Fluorimetric SensoLyte Red Protease Assay Kit (AnaSpec, Fremont, CA, USA), following manufacturer's instructions. Besides the positive and negative controls provided by the kit, two additional negative controls were used, consisting of unrelated proteins produced in *E. coli* and baculovirus systems, respectively, and purified using the same protocols than those used for TMPRSS4.

Production and characterization of TMPRSS4 rabbit polyclonal serum

Purified recombinant TMPRSS4 (1-437) protein expressed in baculovirus system was used to immunize a female New Zealand White rabbit (New Zealand White, IQGsb: MZWR-MDL) in order to obtain specific polyclonal sera (ING-pAb). The rabbit received humane care in accordance with the guidelines for the accommodation and care of animals used for experimental and other scientific purpose according to Animal Welfare Laws (National Laws RD 1201/2005). Aliquots containing 800 µg of recombinant protein in 800 µl of PBS were mixed with an equal volume of Freund's complete adjuvant for the first injection and Freund's incomplete adjuvant for boosters. All injections were administered intramuscularly in the rear legs. Injections were repeated 6 times at 2-week intervals. The total serum immunoglobulin Gs (IgGs) were purified using protein G, following manufacturer's instructions (GE Healthcare Life Science, Uppsala, Sweden). The purified IgGs were dialyzed against PBS, diluted with glycerol (1:1), and stored at -80°C. The specificity of the polyclonal serum was determined by indirect ELISA (i-ELISA) and Western blot analysis using the recombinant proteins and lung cancer cell extracts as antigens. For the i-ELISA, recombinant TMPRSS4 (1-437) was used to coat 96-well Maxisorp plates at 0.5 µg/well. After overnight (ON) incubation at 4°C, wells were blocked with Stabilzyme Select (SurModics Inc.,MN, USA) for 1h at RT. Several concentrations of IgGs were made in 0.05% Tween 20 and 0.35M NaCl in PBS and added to the wells, which were then incubated 1h at 37°C. Bound antibodies were detected by incubation with peroxidase-labelled anti-rabbit IgG (Sigma-Aldrich) (1:5000 dilution). After 1h at RT, the substrate (TMB-MAX Neogen Corporation, Lexington, KY) was added. Sulphuric acid (0.5 M) was used as the stop solution and the absorbance was measured at 450 nm in a Multiscan Ascent ELISA reader.

Additionally, rabbit anti-TMPRSS4 IgGs were peroxidase-labelled (IgGs-HRP) according to the method described by Nakane and co-workers (Nakane and Pierce, 1966) for its further characterization. To test binding efficiency of this conjugated antibody by ELISA, TMPRSS4 (1-437) was used at 0.5 µg/well as coating in 96-well plates, and IgGs-HRP were applied at serial dilutions in 0.05% Tween 20 and 0.35M NaCl in PBS. After 1h at RT, the reaction was developed as described before.

DAS-ELISA

Microtiter plates were coated with 1 µg/well of purified rabbit anti-TMPRSS4 IgGs diluted in sodium carbonate buffer (pH 9.6) and incubated ON at 4°C. After the plates were blocked with Stabilzyme Select (SurModics Inc.,MN, USA) for 1 h at RT, the recombinant TMPRSS4 (1-437) and the negative control proteins diluted in 0.05% Tween 20 in PBS were added to the plates and then incubated 1 h at 37°C. The IgGs, conjugated with HRP and diluted at 1:8000 in PBS-0.05% Tween 20, were used for antigen detection by incubation for 1 h at RT. TMB peroxidase substrate was added, and the reaction was stopped by addition of 0.5 M sulphuric acid.

Cancer cells and modification of endogenous TMPRSS4 levels

The following lung cancer cells were used: A549 and H157, both of them with low endogenous TMPRSS4 mRNA levels; H358 and H441, cell lines with high endogenous TMPRSS4 levels (Larzabal et al., 2011).

The full-length *TMPRSS4* gene was subcloned from the pDONR223 into the pBABE-puro expression vector (Addgene, Cambridge, MA, USA). The AmphoPack-293 packaging cell line (Clontech, Mountain View, CA, USA) was cultured with DMEM (Invitrogen) and transfected with Lipofectamine™ 2000 (Invitrogen) using either the empty plasmid (p-BABE) or the plasmid containing TMPRSS4 (pB-TMP4). The A549 cell line was infected with viral particles plus 8 µg/ml of polybrene (Sigma-Aldrich) and vector-containing cells were selected with 1 µg/ml of puromycin (Invitrogen).

H358 and H441 lung cancer cells were transfected with the pSilencer vector (Invitrogen) containing a shRNA sequence that inhibits TMPRSS4 expression, as we have published previously (Larzabal et al. 2011). Protein expression levels were tested by Western blot.

Western blot in cancer cells and recombinant proteins

Cancer cells were lysed and proteins were extracted with RIPA buffer. Western blots were performed as

previously described (Larzabal et al., 2013). Briefly, protein concentrations were determined with the BCA Protein Assay Kit (Thermo Scientific, Bremen, Germany). After electrophoresis using NuPage 12% Bis-Tris gels (Invitrogen), proteins were transferred to 0.22 µm nitrocellulose membranes (Bio-Rad, Richmond, CA, USA). The membranes were placed in blocking solution (5% non-fat dry milk-PBS-Tween-20) for 1 h at RT and then incubated ON with the primary specific antibodies: ProteinTech-pAb (0.13 ng/µL) or ING-pAb (0.26 ng/µL) in 5% milk-PBS-Tween-20. After incubation with an anti-rabbit HRP-conjugated secondary antibody (GE Healthcare, UK) at 1:2000 dilution in 5% milk-PBS-Tween-20, the Lumi Light Plus chemiluminiscence kit (Roche) was used for visualization of the bands.

Western blots were also carried out by electrophoresing the recombinant protein preparations on SDS-polyacrylamide gels under reducing conditions. Detection of the antigens was carried out by incubation an anti-histidine monoclonal antibody (anti His-Mab) or the ING-pAb anti-TMPRSS4 as primary antibodies, and polyclonal HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies.

Tissue MicroArrays (TMA) and tissues from xenografted cancer cells

The following tissue microarrays were used in the present study: a) a colorectal cancer TMA developed at the Department of Pathology, Hospital Marques de Valdecilla. This TMA contained 41 cases of pT1-pT2 colorectal tumors; b) a Non-Small Cell Lung Carcinoma (NSCLC) TMA that included 74 samples (42 adenocarcinomas and 32 squamous cell carcinomas) developed at the Program of Solid Tumors, CIMA of the University of Navarra, Pamplona, Spain; c) a series of lung cancer TMAs from the Gregorio Marañon Hospital, Madrid, Spain, which included Large Cell Carcinomas (n=50 cases); typical carcinoids (n=40 cases); atypical carcinoids (n=10 cases); tumorlet carcinoids (n=24 cases), and Small Cell Lung Carcinomas (SCLC, n=32 cases); d) an osteosarcoma TMA (n=23 samples) kindly donated by Dr. Ana Patiño (CIMA) plus 5 cases of high grade sarcomas (Clinica Universidad de Navarra).

Regarding tissues from xenografts, H358 lung cancer cells ($n=5x10^5$) were xenotransplanted into mice through tail vein injection and, 35 days later, mice were sacrificed and tumor-bearing lungs were fixed in formaldehyde, embedded in paraffin and routinely processed for histology.

Immunohistochemistry and semiquantification of the signal

Sections were deparaffinized, hydrated, and incubated for 10 min with 3.3% H_2O_2 in water to block endogenous peroxidase. Antigen retrieval was done in a microwave oven with 10 mM citrate buffer at pH 6. Primary antibodies (either ProteinTech-pAb or ING-pAb) were used at 2.6 ng/µL dilution in Dako Real Antibody diluent. Slides were then incubated with the Advance™ detection system (Dako) and peroxidase activity was developed with DAB (3,3'-diamino-benzidine; Dako). Slides were then counterstained with haematoxylin, dehydrated and cover-slipped with DPX mounting medium (VWR, Soulbury, UK). Images were captured with Leica Aperio CS2.

TMPRSS4 immunostaining was blindly evaluated by two observers, taking into account both extension and intensity of the staining, in accordance with previous reports (Pajares et al., 2012). Extension was expressed as the percentage of positive cells, and intensity was classified as negative (0), weak (1), moderate (2), and strong (3). Samples were grouped according to the H-score as: negative (H-score <20), low-medium (H-score 20-100), high (H-score 100-120) or very high (H-score >120).

Statistical methods

Data were analyzed with GraphPad Prism 5 software (GraphPad). Spearman's analysis was performed to correlate expression of TMPRSS4 between the two antibodies used in this study. Values are expressed as means ± SD.

Results

Expression of TMPRSS4 recombinant proteins

The complete TMPRSS4 (1-437 aa) protein was expressed and produced in *E. coli* (52 kDa) and BEVS (54 kDa). A version of the gene without the transmembrane region was generated by PCR and cloned into the *E. coli* transfer vector pDEST17-GW to generate the truncated version of TMPRSS4 (54-434 aa) recombinant protein with a molecular weight of 49 kDa. Finally, the catalytic region of TMPRSS4 (from 205 to 434 aa) was amplified and cloned into the pDEST17-GW and pAcHLTA-GW transfer vectors, for its expression in *E. coli* and BEVS systems, respectively. The expected molecular weight of these two proteins was 28 and 30 kDa, respectively. The full length and truncated forms of TMPRSS4 proteins were successfully expressed and purified using both BEVS and *E. Coli* expression systems, as shown by SDS-PAGE and Coomasie blue staining (Fig. 2A). Since the proteins had a 6 amino acid histidine tag, we next characterized the proteins by Western blot using an anti-His antibody. Bands of the expected molecular mass were found for all the proteins analysed (Fig. 2B) The nature of the recombinant proteins was also examined by Western blot using the rabbit polyclonal anti-TMPRSS4 (ING-pAb). As shown in Fig. 2C, the expected specific bands were observed for all the proteins generated. Recombinant proteins that only contained the catalytic domain were substantially less immunoreactive than the other recombinant proteins (Fig. 2C).

Expression levels in both systems were high, ranging from 1 mg/ml to 1.5 mg/ml, as estimated by densitometric assays and comparison with known quantities of BSA in SDS-PAGE gels, and the purity of the proteins was higher than 90%. This shows a high efficiency in the production and purification processes.

Enzymatic activity of TMPRSS4 recombinant proteins

To confirm that the purified and refolded TMPRSS4 recombinant proteins had serine protease activity, the Fluorimetric SensoLyte™ Red Protease Assay Kit was used. Fig. 2D shows that the purified proteins displayed protease activity, as they generated efficient fluorescence upon reaction with the substrate provided by the kit (casein). Protease activity of all the TMPRSS4 recombinant proteins showed a similar pattern of activity and reached an RFU close to the positive control (trypsin) provided by the Kit. Negative controls consisted of unrelated proteins without protease activity, produced and purified in the same conditions than TMPRSS4.

Characterization of the rabbit polyclonal antibody directed against TMPRSS4

The IgGs of the ING-pAb were purified and evaluated by indirect ELISA after coating the plates with 0.5 µg/well recombinant TMPRSS4 (1-437 aa). As negative control, plates were coated with different proteins, including lysozyme, fibrinogen, albumin, ovalbumin and human IgG (data not shown). Binding of IgGs was found to be specific and the titer, expressed as the inverse of the highest dilution that still gives a positive result, was 1:16,000 (Fig. 3A). Then, IgGs were conjugated with peroxidase and tested by ELISA using

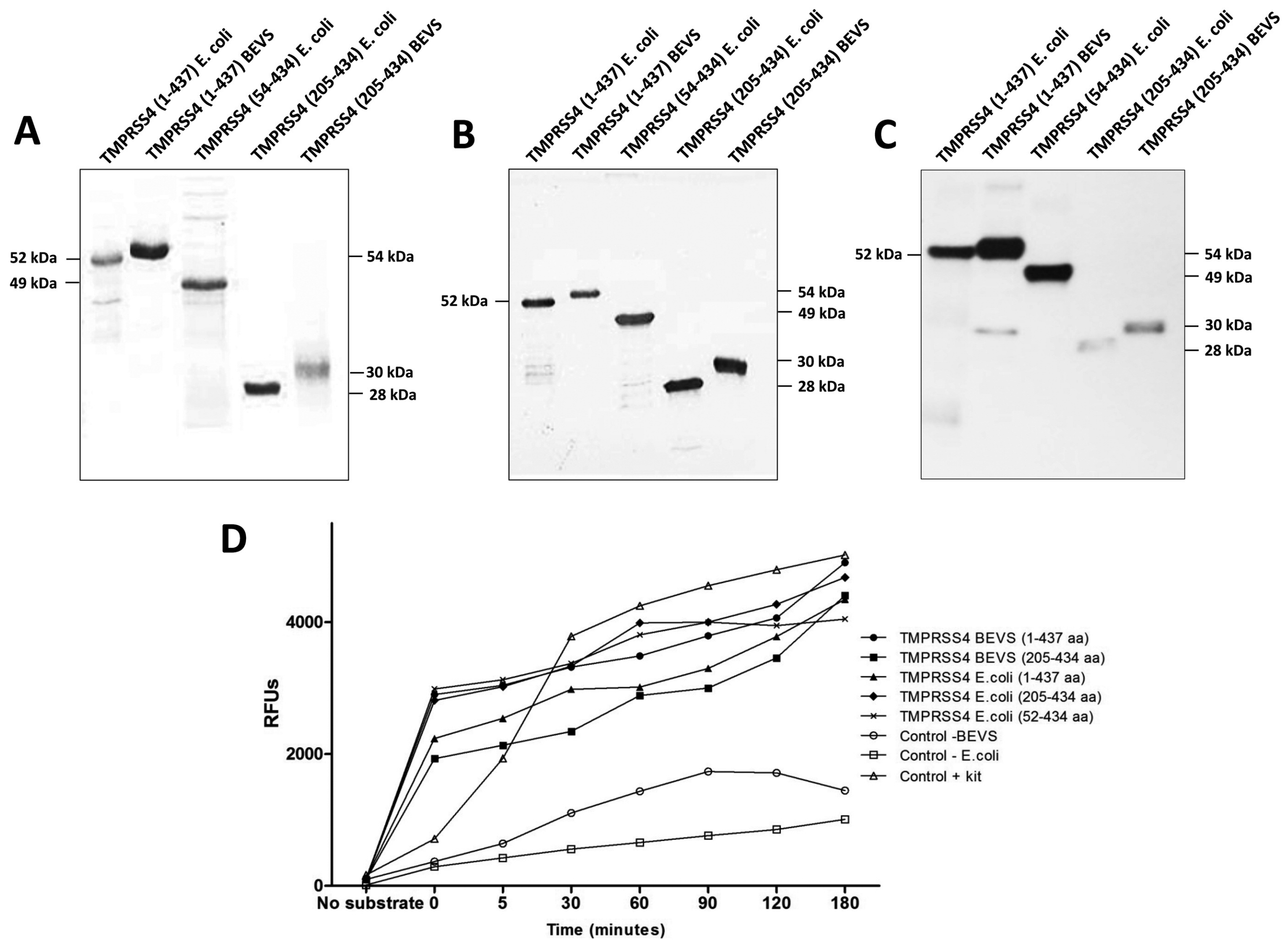

Fig. 2. Analysis of the recombinant proteins by SDS-PAGE, protease activity and Western blot. **A.** Coomassie blue staining. **B.** Western blot using anti-histidine (His) monoclonal antibody. **C.** Western blot using ING-pAb anti-TMPRSS4. **D.** Activity of the full-length and truncated recombinant proteins produced in *E. coli* or baculovirus systems.

the same antigen. The titer of the IgGs-HRP was estimated to be 1:64,000 (Fig. 3B). The cut-off value was calculated as twice the mean absorbance value of the negative control.

The DAS-ELISA was set up using the recombinant protein as the antigen, IgGs as the capture antibody and different dilutions of the IgGs labelled with peroxidase. This assay was able to detect up to 20-30 ng/ml (2-3 ng/well) of recombinant protein. The results are shown in Fig. 3C.

The ability of the ING-pAb to bind TMPRSS4 in cancer cell extracts was further investigated by Western blot analysis. The full length TMPRSS4 recombinant protein was used as control, for both ING-pAb and Pro Tech-pAb antibodies (Fig. 4A). A panel of cell lines with different TMPRSS4 expression levels were examined: H358 and H441 (cells with high TMPRSS4 expression); H358shTMP and H441shTMP, which correspond to clones with depleted levels of this protein, previously characterized by our group (Larzabal et al., 2011); A549 and H157 (cells lacking endogenous TMPRSS4); and A549TMP4, a previously characterized A549 clone with TMPRSS4 overexpression (de Aberasturi et al., 2016). Fig. 4B shows the expected band for cells H358, A549TMP and H441 when using ING-pAb. Levels for H358shTMP and H441shTMP were lower than those found for their respective controls. As expected, no band was observed for A549 and H157 cells. The same blots were performed with the Pro Tech-pAb, which showed two bands within the range of the expected size (Fig. 4B).

Immunohistochemical staining was also carried out in lung tumors orthotopically generated by injection of H358 cells into the tail vein. Fig. 4C shows that tumor cells were specifically labelled by both antibodies. However, ING-pAb gave a much stronger staining than that found for Pro Tech-pAb at the same concentration (2.6 ng/μL).

Validation of immunostaining in colon cancer samples and identification of novel tumors that express TMPRSS4

To further evaluate ING-pAb immunostaining we analyzed a TMA containing 41 colon cancer samples. Four samples (9.8%) were negative for TMPRSS4 and weak H-score was obtained in 8 tumors (19.5%). Six samples (14.6%) showed a high H-score and 23 (56%) a very high H-score (>120). The signal was found in the cytoplasm and membrane of tumor cells. This pattern is similar to that described in colon cancer using our reference Pro Tech-pAb (Kim et al., 2010). We also performed immunohistochemistry comparing the staining pattern of both ING-pAb and Pro Tech-pAb in a TMA containing 74 cases of NSCLC. As shown in Fig. 4D, there was a very significant correlation ($r=0.65$; $p<0.0001$) between TMPRSS4 levels analysed by ING-pAb and by Pro Tech-pAb.

We then evaluated immunoreactivity of ING-pAb in the whole set of lung cancer TMAs and studied the H-

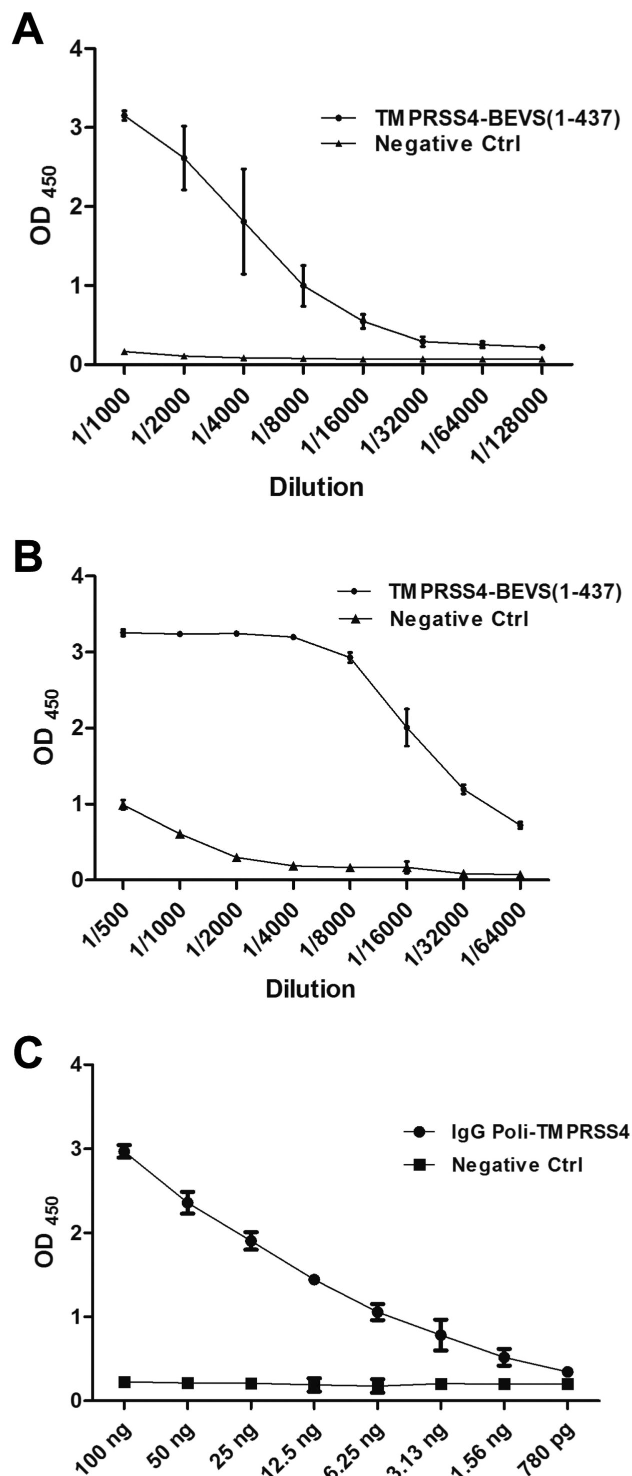

Fig. 3. Characterization of IgGs against TMPRSS4. Indirect ELISA using IgGs **(A)** and IgGs-HRP **(B)** in plates coated with 0.5 µg/well recombinant TMPRSS4 (1-437 aa) or lysozyme as negative control. **C.** TMPRSS4 detection by DAS-ELISA. Purified IgGs were used as the capture antibody. Serial dilutions of TMPRSS4 and negative control were added to the plates and then detected with IgGs-HRP (1:8000). The absorbance at 450 nm was plotted against the amount of protein expressed in ng.

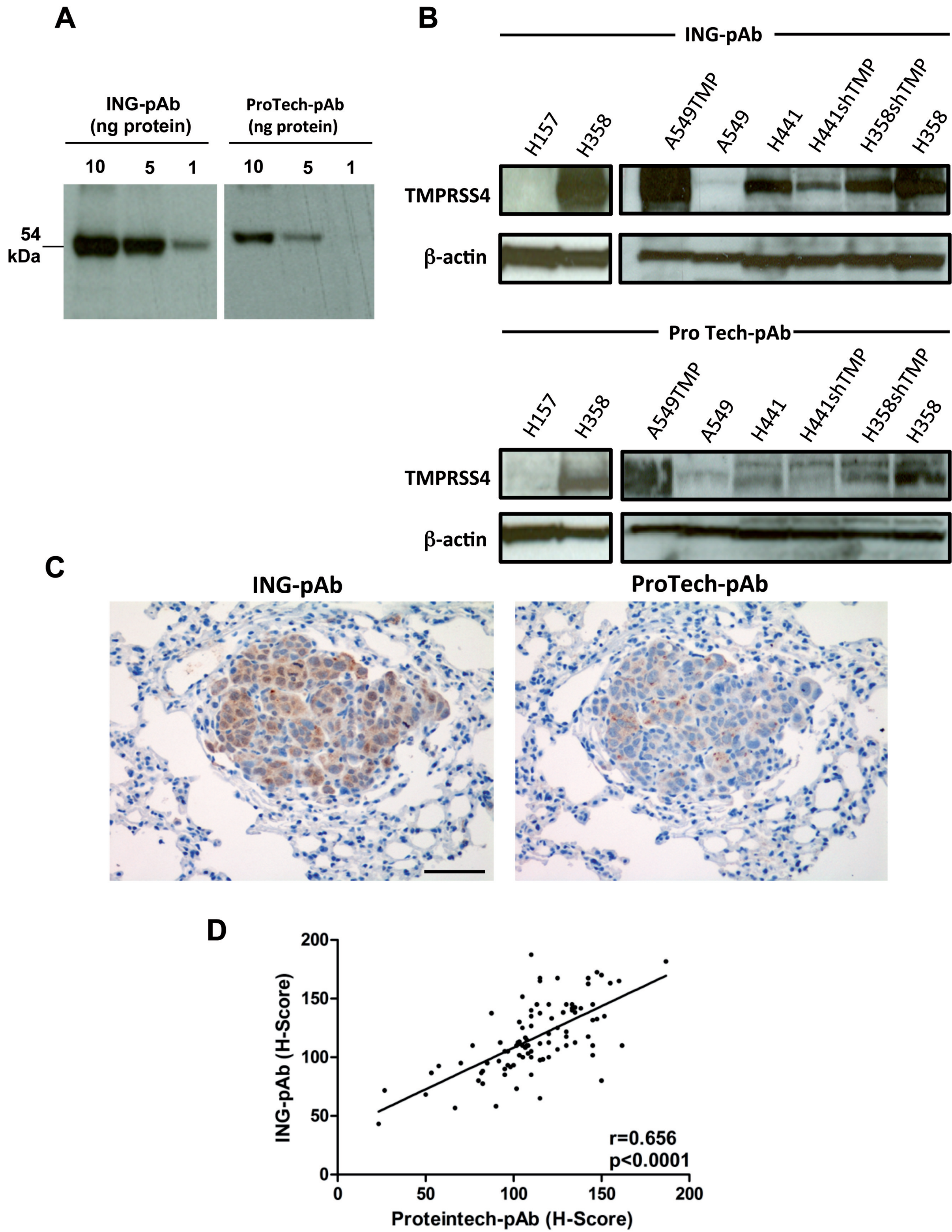

Fig. 4. Characterization of the ING-pAb by Western blot (in cell extracts) and immunohistochemistry. **A.** Western blot using different concentrations of the recombinant protein TMPRSS4 (1-437) produced in BEVS and incubated with either ING-pAb or Pro Tech-pAb antibodies. **B.** Western blot to show TMPRSS4 in cell extracts, using either ING-pAb or Pro Tech-pAb. TMPRSS4 protein levels were the expected ones according to expression of each of the cell lines: No band was found for H157 and A549 cells; a strong band corresponding to ~50 kDa was observed for A549TMP (TMPRSS4-overexpressing cell), H441 and H358; cells with depleted TMPRSS4 expression (H441shTMP) and (H358shTMP) showed a band with lower intensity than the one found for the control cells. The same pattern was observed for both antibodies. **C.** Immunohistochemsitry for TMPRSS4 in H358 cells orthotopically xenografted into nude mice. The signal was stronger when the ING-pAb was used, as compared to that found for Pro Tech-pAb antibody at the same concentration. **D.** Correlation analysis of H-score found for ING-pAb and Pro Tech-pAb in lung cancer specimens. Scale bar: 50 µm.

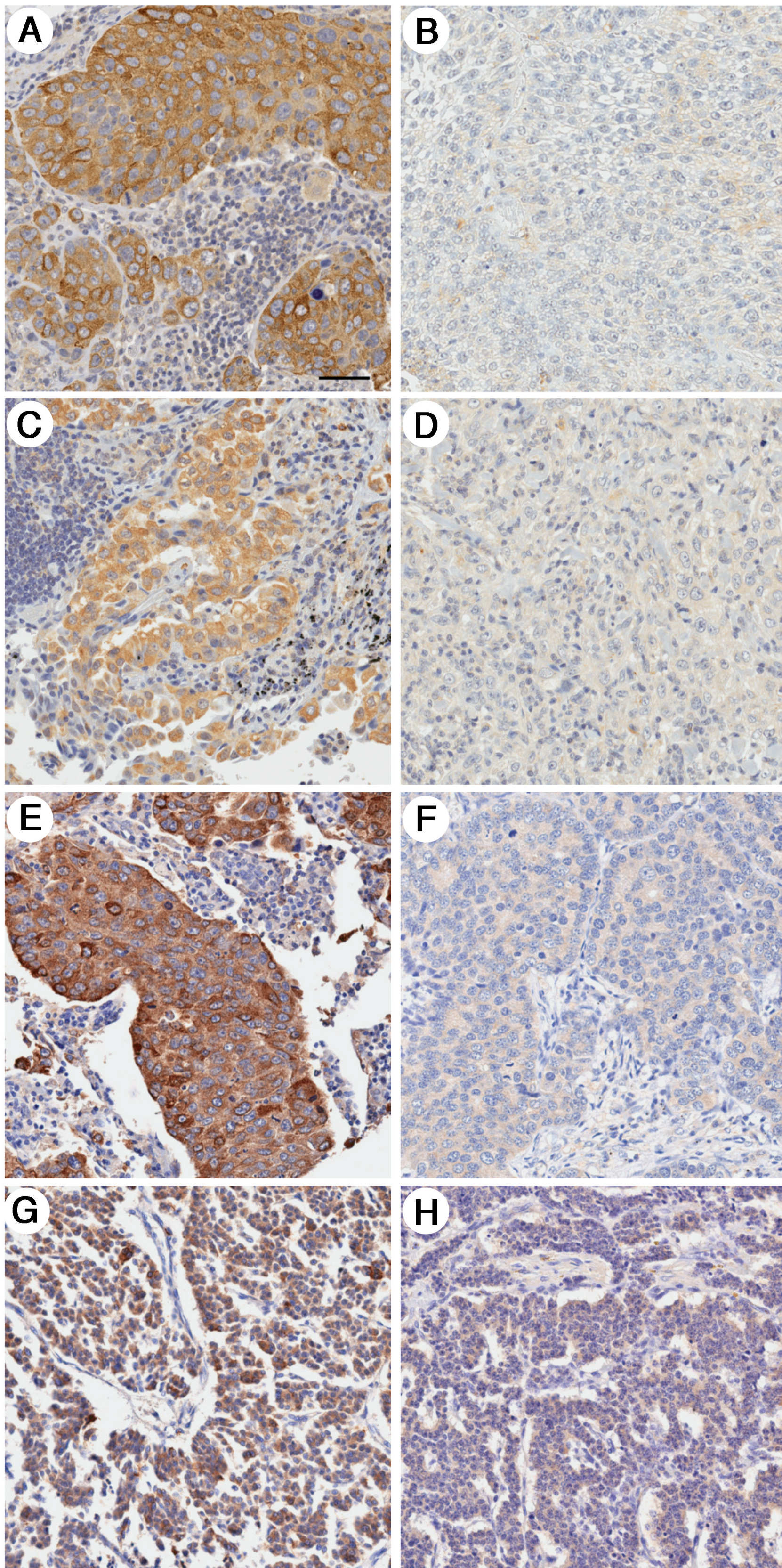

Fig. 5. TMPRSS4 immunostaining patterns in different lung cancer types. Examples of strong staining in squamous cell carcinoma **(A)**, adenocarcinoma **(C)**, Large Cell Carcinoma **(E)**, and atypical cell carcinoid **(G)**. Examples of weakly or no stained samples of each of these lung cancer types are shown in **B**, **D**, **F**, and **H**, respectively. Scale bar: 50 µm.

Table 2. H-score data for TMPRSS4 staining in the different lung cancer types. Values were subclassified in 4 groups depending on the H-score range: <20 was considered negative; 20-100: low-medium; 100-120: high; >120: very high. The number and percentage of cases in each class is indicated.

Histological type	Very high (>120), n (%)	High (100-120), n (%)	Low-Medium (20-100), n (%)	Negative (<20), n (%)
NSCLC				
Adenocarcinomas (n=42)	15 (35.7)	10 (23.8)	17 (40.5)	0 (0)
Squamous cell carcinomas (n=32)	18 (56)	6 (19)	8 (25)	0 (0)
Large cell carcinomas (n=50)	4 (8)	3 (6)	18 (36)	25 (50)
SCLC (n=32)	0 (0)	0 (0)	16 (50)	16 (50)
Others				
Typical Carcinoids (n=40)	18 (45)	18 (45)	5 (10)	0 (0)
Atypical Carcinoids (n=10)	4 (40)	2 (20)	3 (30)	1 (10)
Tumorlet carcinoids (n=24)	1 (4.2)	1 (4.2)	6 (25)	16 (66.6)

score according to 4 categories (Table 2). Fig. 5 illustrates examples of immunostaining using ING-pAb in tumors with different histology and staining pattern. In all positive cases, staining was observed specifically in malignant cells. A high percentage of adenocarcinomas and squamous cell carcinomas (the most frequent NSCLC types) showed a high (H-score 100-120) or very high (H-score >120) score (56% for squamous tumors and 35.7% for adenocarcinomas, Table 2). All typical carcinoids were positive for TMPRSS4 and 45% of them showed a very high score. On the contrary, only a small percentage of large cell carcinomas (8%) had a score higher than 120, but these tumors were strikingly positive for TMPRSS4 (Fig. 5). Forty percent of atypical carcinoids were also positive, whereas the majority of tumorlet carcinoids were negative. Regarding SCLC, 50% were weakly positive and the rest of them had no staining (Table 2).

Concerning sarcomas, all the cases (n=18 osteosarcomas and 5 high grade sarcomas) were negative for TMPRSS4. In osteosarcomas, only osteoclasts and ~25% of osteoblasts were positive (results not shown).

Discussion

TMPRSS4 has been suggested as a promising biomarker and therapeutic target in several solid tumors, such as lung (Larzabal et al., 2011), breast (Cheng and Hong Kong, 2013a,b), colon (Kim et al., 2010) and liver (Wang et al., 2015). This protease promotes tumor invasion and accelerates metastasis (de Aberasturi and Calvo, 2015). High levels in tumors have been associated with decreased disease-free survival and overall survival, which makes TMPRSS4 a promising prognostic factor. Although several antibodies are now commercially available for immunohistochemistry, some of them cannot be considered as appropriate tools for clinical use.

Based on this fact, we decided to develop an antibody against TMPRSS4 that could be accurately used by Western blot and immunohistochemistry in clinical samples and to test its performance in comparison to the antibody used in the majority of clinical studies (Cheng and Hong Kong, 2013a,b; Dai et al., 2013; Luo et al., 2013). Both antibodies were able to specifically bind the native protein by Western blot in cell lines, following the expected pattern of basal expression and changes of such levels through knock-down/overexpression systems. The pattern of immunostaining in xenografts and human cancer specimens was also similar. However, ING-pAb was more sensitive than our reference antibody from Protein Tech (Pro Tech-pAb). We also validated with the ING-pAb antibody that the pattern of TMPRSS4 expression in colon cancer samples was coincident with the one previously published (Kim et al., 2010). Of note, the H-score for both ING-pAb and Pro Tech-pAb correlated very significantly in a cohort of lung cancer patients.

Among the lung tumors analysed, squamous cell carcinomas showed the highest percentage of cases with H-score>120: 56%. In contrast, only 35% of ADC presented this score. This is in keeping with our previous findings that revealed ~2.5-fold higher levels in SCC than in ADC (Larzabal et al., 2011). Both typical and atypical carcinoids also showed a high percentage of cases with H-score>120 (45% and 40%, respectively). In these tumor types, morphology alone has no prognostic or predictive value, and expression of somatostatin receptors and proteins of the mTOR pathway is relevant for their molecular characterization and management of the patients (Righi et al., 2014). It has been suggested that delineation of a more informative molecular map of active pathways in these tumors will help establishing new therapeutic approaches. As a substantial percentage of typical carcinoids display either high or very high H-score, we suggest that TMPRSS4 may play a role in the biology of these tumors. Future studies should investigate in more depth whether this expression is related to relapse, survival or therapy response.

Of particular interest is the identification of a subset of Large Cell Carcinomas (LCC) strongly positive for TMPRSS4. LCC is an uncommitted histopathological term that describes pulmonary carcinomas having undifferentiated features, without neuroendocrine, squamous or glandular differentiation, as well as no specific clinical characteristics (Pelosi et al., 2015). Management of these patients is also cumbersome. Different studies have suggested the need for recategorizing this tumor entity based on molecular profiling and, in this sense, TMPRSS4 could define a subgroup of LCC with particular biological properties. Whether high TMPRSS4 expression in LCC is associated with an aggressive behaviour, which may help stratifying these patients for certain treatments, should be determined in future studies.

The increasing number of studies on TMPRSS4 suggests that this serine protease may become a cancer

target. The fact that this protein is highly expressed in different solid tumor types in association with poor prognosis, its involvement in metastasis, and its druggability (Dawelbait et al., 2007; Kang et al., 2013; Hamamoto et al., 2015), encourages the development of specific inhibitors. In order to analyze TMPRSS4 blockade it is necessary to establish biochemical systems to test its activity. We have described here the successful cloning, purification and protease activity of either the full length protein or fragments that contain the catalytic activity, using both *E. coli* and baculovirus systems of expression. Although the different proteins generated had similar activity regardless of the expression system, glycosylation will be lacking in *E. coli*, which may be problematic for certain properties of the protein.

In conclusion, we have developed new useful biological tools for the evaluation of expression and activity of the prometastatic protein TMPRSS4. Protein expression analyses led us to the identification of a subset of lung cancer types where this protein may have a potential clinical value. Future studies using these tools should determine whether TMPRSS4 should be considered a biomarker for aggressive tumors.

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