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DEVELOPMENT OF NOVEL MONOCLONAL ANTIBODIES AND IMMUNOASSAYS FOR SENSITIVE AND SPECIFIC DETECTION OF SULF1 ENDOSULFATASE

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Abstract

BACKGROUND—Cell-surface heparan sulfate proteoglycans (HSPGs) function as receptors or co-receptors for ligand binding and mediate the transmission of critical extracellular signals into cells. The complex and dynamic modifications of heparan sulfates on the core proteins are highly regulated to achieve precise signaling transduction. Extracellular endosulfatase Sulf1 catalyzes the removal of 6-O sulfation from HSPGs and thus regulates signaling mediated by 6-O sulfation on HSPGs. The expression of Sulf1 is altered in many cancers. Further studies are needed to clarify Sulf1 role in tumorigenesis, and new tools that can expand our knowledge in this field are required.

METHODS—We have developed and validated novel SULF1 monoclonal antibodies (mAbs). The isotype and subclass for each of these antibodies were determined. These antibodies provide invaluable reagents to assess SULF1- tissue and blood levels by immunohistochemistry and ELISA assays, respectively.

RESULTS—This study reports novel mAbs and immunoassays developed for sensitive and specific human Sulf1 protein detection. Using these SULF1 mAbs, we developed an ELISA assay to investigate whether blood-derived SULF1 may be a useful biomarker for detecting cancer early. Furthermore, we have demonstrated the utility of these antibodies for Sulf1 protein detection, localization, and quantification in biospecimens using various immunoassays.

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Credit author statement:

None

CONCLUSIONS—This study describes novel Sulf1 mAbs suitable for various immunoassays, including Western blot analysis, ELISA, and immunohistochemistry, which can help understand Sulf1 pathophysiological role.

GENERAL SIGNIFICANCE—New tools to assess and clarify SULF1 role in tumorigenesis are needed. Our novel Sulf1 mAbs and immunoassays assay may have utility for such application.

Keywords

SULF1; extracellular sulfatase; heparan sulfate; monoclonal antibodies; immunoassays; ELISA; western blot; Immunohistochemistry; plasma; biomarker; cancer

INTRODUCTION

Much interest has been focused on biomarker discovery and their clinical utility. It is anticipated that cancer biomarkers will be critical for advances in early diagnosis, stratification of patients for appropriate therapies, and monitoring response to therapy. Numerous potential protein biomarkers have been identified in cancer, including carcinoembryonic antigen (CEA), CYFRA21–1, plasma kallikrein B1 (KLKB1), and neuron-specific enolase [1–3]. Despite these studies, currently, there are no reliable biomarkers for cancer detection in clinical use. One reason for these failures may be that when applied to a heterogeneous cancer population, the biomarker lacks the required sensitivity and specificity for a diagnostic marker in the absence of functional relevance.

The extracellular sulfatases (SULF1 and SULF2) are overexpressed in a wide assortment of human cancers, and SULF2 has been shown to drive carcinogenesis in particular NSCLC, malignant astrocytoma, and hepatocellular carcinoma [4, 5]. The SULFs are unique as they regulate the binding of numerous signaling molecules by editing essential sulfation modification on their substrate, heparan sulfate proteoglycans (HSPGs). HSPGs consist of core proteins modified by the covalent addition of HS chains containing variably sulfated repeating disaccharide units [6]. HSPGs carry out enumerable signaling functions by binding of their sulfated chains to diverse ligands, such as cytokines, growth factors, morphogens, chemokines, and morphogens. These protein interactions largely depend on the pattern and sulfation modifications density, with 6-O-sulfation of glucosamine (6OS) known to be essential for many ligand interactions [5]. SULFs provide a novel mechanism for the regulation of HSPG-dependent signaling by removing 6OS. The SULFs remove 6OS from HSPGs and promote critical signaling pathways by mobilizing ligands from HSPG, and thus liberate the ligands for binding to signal transduction receptors [7–9].

The SULFs are extracellular enzymes and are both bound to the cell membrane and secreted. SULFs and their substrates are present in the extracellular milieu, and therefore have great potential as novel blood biomarkers for early detection of cancer.

For SULF1, the focus of this present study, the cell surface interaction is mediated by binding a hydrophilic domain in the catalytic site to heparan sulfate chains [10]. The hydrophilic domain is obligatory for heparan sulfate endosulfatase activity, apparently

through its oriented presentation of HS chains to the enzyme's catalytic site in a processive manner [11].

Previous reports suggested a tumor suppressor for SULF1 in a subset of tumors [12–15]. SULF1 transcript is downregulated in ovarian, breast, kidney, pancreatic and hepatocellular cancer cell lines and primary ovarian tumors [16]. The lower expression level of SULF1 transcript was also observed in lung cancer cell lines compared to healthy lung cells [17]. Overexpression of SULF1 in SKOV3, an ovarian cancer cell line lacking detectable SULF1 mRNA, reducing cell proliferation and inhibited phosphorylation of EGFR induced by heparin-dependent EGF and the ERK phosphorylation induced by FGF2 [16]. Also, overexpression of SULF1 sensitized SKOV3 cells to cisplatin [16]. In vivo, SULF1 overexpressing clones of MDA-MB-468, a breast cancer cell line, resulted in smaller tumor sizes and reduced angiogenesis than the parental cells in xenograft model [18].

Conversely, broader analyses of public gene microarray data showed that the SULF1 gene is upregulated in most cancer types investigated, including breast, lung, kidney, and pancreatic cancers [5, 19, 20] and T prolymphocytic leukemia, acute myeloid leukemia and renal carcinoma [4]. Moreover, our previous investigation showed that SULF1 and SULF2 transcripts are overexpressed in a human non-small lung tumor (adenocarcinomas and squamous carcinomas) compared to adjacent healthy lung tissues [20]. In urothelial carcinoma, higher Sulf1 expression level is associated with higher histological grade and poorer outcome [21]. SULF1 overexpression was also associated with poor prognosis in lung cancer but not in breast cancer even though the expression of SULF1 was upregulated [4]. To date, the role of Sulf1 in tumorigenesis remains unclear. Thus, more investigations are required to determine the expression of SULF1 during tumor progression and its contribution to carcinogenesis.

Here, we report new reagents developed in our laboratory that can be applied to Sulf1 studies. We have developed novel Sulf1 mouse monoclonal antibodies suitable for a variety of immunoassays, including Western blot analysis, enzyme-linked immunosorbent assay (ELISA) immunohistochemistry (IHC), which can help in the understanding of the pathophysiological role of Sulf1. We have demonstrated that both western blot assays and ELISAs assays are applicable to evaluate SULF1 levels in plasma.

MATERIALS AND METHODS

Hybridoma culture and antibody production

17 clones of hybridoma cells were obtained from Abmart (Shanghai, China) and were cultured in DMEM/F-12 (50%/50%) media supplied with 15% of fetal bovine serum. For small scale antibody production, cells were grown in T150 flasks in DMEM/F-12 (50%/50%) media supplied with 15% of Ultra-low IgG fetal bovine serum until 90% of the cells were dead. Antibodies were harvested from the culture media by centrifuging at 1000g for 5 minutes and filtered through 0.45 um filter. For large scale antibody production, cells were first adapted to grow in serum-free Hybridoma-SFM (Thermo Fisher Scientific Inc., Waltham, MA) and then seeded to the lower chamber in a CELLine bioreactor (Corning, Corning, NY). The Hybridoma-SFM media added to the upper chamber was renewed

weekly. Antibodies were harvested weekly from the culture media in the lower chamber by centrifuging at 200g for 5 minutes, followed by centrifuging the supernatant at 1000 g for 5 minutes. The supernatant was stored at −80°C for antibody purification.

Antibody purification

Protein G beads (GE Healthcare, Little Chalfont, UK) were packed in Pierce 10 ml centrifuge columns (Thermo Fisher Scientific Inc., Waltham, MA), washed with five times of column volume of Milli-Q water and equilibrated with five times of column volume of calcium and magnesium-free phosphate-buffered saline. Hybridoma culture media harvested from bioreactor were added to the column, followed by washing with ten times of the column volume of calcium and magnesium-free phosphate-buffered saline. Five times of column volume of 10 mM Diethylamine (Sigma-Aldrich, St. Louis, MO) was added to elute bound antibodies. Antibodies were collected from the first two fractions and neutralized with 1N HCl immediately. The eluted antibodies were dialyzed in 10K MWCO SnakeSkin™ Dialysis tubing, 22 mm (Thermo Fisher Scientific Inc., Waltham, MA) against 2 liters of calcium and magnesium-free phosphate-buffered saline for two or three times, and then concentrated using Amicon Ultra-0.5 ml, 30 MWCO centrifuge filter units (EMD Millipore, Darmstadt, Germany).

Antibodies Biotinylation

Antibodies were first filtered with Amicon Ultra-0.5 ml, 30 MWCO centrifuge filter units (EMD Millipore, Darmstadt, Germany) to concentrated antibodies and to remove sodium azide. One to two milligrams of antibodies were subjected to conjugation with biotin using EZ-linked PEG biotinylation kit (Pierce, Thermo Fisher Scientific Inc., Waltham, MA) according to manufacture instruction. Briefly, antibodies were mixed with biotins and incubated on ice for two hours. Free biotins were removed using Zeba desalting column (Thermo Fisher Scientific Inc., Waltham, MA) followed by three rounds of dialysis against calcium and magnesium-free phosphate-buffered saline.

Heparin-capture ELISA

Immulon 2HB plates (Thermo Fisher Scientific Inc., Waltham, MA) were coated with 1 ug/ml heparin-BSA [22] at 4°C overnight. Plates were washed with 200 μl of phosphate-buffered saline supplied with 0.1% Tween 20 (PBST) three times and blocked with 200 μl of 3% Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) in PBST at room temperature for one hour. After three PBST washes, 100 μl of samples were added to wells and incubated at room temperature for one hour. Wells were washed with PBST three times 0.5 μg/ml of biotin-labeled and detection antibodies were added to wells followed by one-hour incubation at room temperature. 100 ng/ml of Horseradish peroxidase (HRP) conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) was added to wells after three PBST washes and incubated at room temperature for 30 minutes. After three 5-minute washes, ultra TMB-ELISA substrate (Thermo Fisher Scientific Inc., Waltham, MA) was added to wells for 20 minutes and 100 μl of 0.2 M H_2SO_4 was added to stop the reaction. The absorbance at 450 nm was read.

Heparin-captured 4-C25 bound Sulf1 ELISA

Immulon 2HB plates (Thermo Fisher Scientific Inc., Waltham, MA) were coated with 1 ug/ml heparin-BSA [22] at 4°C overnight. Samples were incubated with Sulf1 monoclonal antibodies (1 ug antibody per 100 ul of samples) at 4°C overnight. The next day, plates were washed with 200 μl of phosphate-buffered saline supplied with 0.1% Tween 20 (PBST) three times and blocked with 200 μl of 3% BSA (Sigma-Aldrich, St. Louis, MO) at room temperature for one hour. After three 200 μl of PBST washes, sample-antibody mixture was added to wells and incubated at room temperature for one hour. Wells were washed with 200 μl of PBST three times followed by 30 minutes incubation of 100 ng/ml of Horseradish peroxidase (HRP) conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). After three 5-minute washes, 100 μl of ultra TMB-ELISA substrate (Thermo Fisher Scientific Inc., Waltham, MA) was added to wells for 20 minutes and 100 ul of 0.2 M H_2SO_4 was added to stop the reaction. The absorbance at 450 nm was read.

PEI transfection

293T cells were seeded to T150 flasks and cultured in DMEM media until reaching 70–80%. Twenty-five micrograms of the plasmid (pcDNA3.1/Myc-His(−)-HSulf-1 or -HSulf-2) [7] mixed with 1 mg/ml polyehylenimine (PEI) at ratio of 1:3 (DNA:PEI) in 1 ml of Opti-MEM serum-free media. After incubating at room temperature for 15–20 minutes, DNA-PEI mixture was added to cells with gentle mixing. Twenty to twenty-four hours after transfection, cells were rinsed with Opti-MEM once and changed to Opti-MEM for additional culture of 72 hours. Recombinant HSulf1 protein were harvested from culture media by centrifuging at 1000 g for 5 minutes. The supernatant (condition media = CM) was transferred to clean tubes and stored at 4°C for short-term storage or stored at −80°C for long-term storage.

Generation of stable cell lines

Human 293 cells were transfected with pcDNA3 pcDNA3.1/Myc-His(−)-HSulf-1 or -HSulf-2) [7] using PEI reagent. Forty-eight hours after transfection, G418 (Geneticin®) (Thermo Fisher Scientific Inc., Waltham, MA) was added to cells at a concentration of 500 ug/ml. Obtained G418-resistant clones were maintained in G418 media.

SULF1 depletion assay

Mouse IgG, Sulf2 antibody 8G1 and Sulf1 antibody 4-C25 were conjugated to protein G agarose beads (GE Healthcare, Little Chalfont, UK) by incubating antibodies with beads at 4°C overnight. The conjugated antibodies were added to Sulf1 or Sulf2 CM (1 ug of antibody per 100 ul of CM) and rocking at 4°C overnight. The beads were pelleted at 14,000 g at 4°C for 1 minute. The supernatant was used for heparin-captured 4-C25 bound Sulf1 ELISA assay described as above.

Purification of recombinant HSulf1

Culture media from 293T cells transiently transfected with pcDNA3.1/Myc-His(−)-HSulf-1 plasmid was incubated with Ni-NTA agarose (Qiagen, Venlo, Netherlands) in the presence of 15 mM imidazole at 4°C overnight. The mixture was poured into a poly-prep

chromatography column (Bio-Rad, Hercules, CA) and the media were flowed through the column to capture Ni-NTA agarose bound rHSulf1. The beads were washed with three times of column volume of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20, pH 8) and the bound rHSulf1 was eluted with five times of column volume of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 0.05% Tween 20, pH 8). The eluted rHSulf1 was concentrated using Amicon Ultra-0.5 ml, 50 MWCO centrifuge filter units (EMD Millipore, Darmstadt, Germany), washed into phosphate-buffered saline and purified again using HisPur Cobalt purification kit (Thermo Fisher Scientific Inc., Waltham, MA) according to manufacturer instruction. The final eluted fraction was analyzed using gel electrophoresis and subjected to SYPRO® Ruby Protein Gel Stain (Thermo Fisher Scientific Inc., Waltham, MA). The protein was quantified by comparing the band intensity with a BSA standard curve using ImageJ (National Institutes of Health, Bethesda, MD) [23].

Heparin pull-down of Sulfs

Culture media containing recombinant Sulf proteins were rocking with Heparin Sepharose 6 Fast Flow (GE Healthcare, Little Chalfont, UK) at 4°C overnight. Heparin beads were pellet at 3000g for 5 minutes at 4°C and washed with calcium and magnesium-free phosphate-buffered saline twice. The bound proteins were eluted from heparin sepharose by adding bead volume of 2x Laemmli Sample Buffer (Bio-Rad, Hercules, CA) and boiling at 95–100°C for 5 minutes. After centrifuging at 14,000 rpm for one minute, the supernatant was subject to Western blot analysis.

Western blot assays

Samples were loaded to 7.5% 1x TGX gels (Bio-Rad, Hercules, CA) and separated using gel electrophoresis. Proteins were transferred to Millipore Immobilon PVDF Transfer Membranes (EMD Millipore, Darmstadt, Germany) and blocked with 5% non-fat milk Blocker (Bio-Rad) in Tris-buffered saline supplied with 0.1% Tween 20 (TBST) for one hour. Primary antibodies in 5% non-fat milk in TBST (ascites: 1 to 200; purified antibodies: 1 ug/ml) were added to blots and incubated at 4°C overnight. Blots were washed three times with TBST for 10 minutes and incubated with HRP-conjugated goat anti-mouse IgG (1 to 5000 in 5% non-fat milk in TBST) (Jackson ImmunoResearch, West Grove, PA) for one hour. After three TBST washes, membranes were incubated with SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific Inc., Waltham, MA) for 5 minutes and exposed to X-ray films.

Plasmas from early-stage NSCLC and COPD Patients and healthy individuals

Under the UCSF institutional review board approval, plasma samples were obtained from the thoracic tissue bank in the UCSF Thoracic Oncology lab, including a cohort of patients (before surgical resection) of newly diagnosed with histologically confirmed early-stage non-small cell lung carcinoma (NSCLC), who underwent surgery at UCSF. Plasmas from healthy individuals and COPD Patients were obtained from Vital Products, Inc. (Boynton Beach, FL).

Immunohistochemistry

Under the UCSF institutional review board approval, archived paraffin-embedded tissue blocks were obtained for two cases of squamous lung carcinoma from the UCSF thoracic tissue bank in the Thoracic Oncology lab. Tissue sections (5 micron thickness) were deparaffinized (EZ-Prep, Ventana Medical Systems, Roche,Basel, Switzerland) at 75 °C, followed by 30 min antigen-retrieval (Cell Conditioning 1, pH 8.5, Ventana Medical Systems) at 95–100 °C. 3% H2O2 (Thermo Fisher Scientific Inc., Waltham, MA) were used to reduce background staining for 8min. Antibodies were incubated at room temperature for 32 min, at 1:1000 dilutions. Antibody staining was developed using the UltraView Universal DAB detection system (Ventana Medical systems), and accompanied by hematoxylin counterstain.

Statistical analysis

All numerical data were calculated as means ± standard deviations (SD). Differences between groups were compared with a Student's t–test. A p value ≤ 0.05 was considered significant.

RESULTS

Production and evaluation of SULF1 Monoclonal Antibodies

To generate Sulf1 specific monoclonal antibodies, we used Abmart contract research services (Abmart, NJ). Briefly, the full-length SULF1 amino sequence (Figure 1a) was subject to a rigorous analysis using a proprietary algorithm. This method (named SEALTM) aims to identify protein fragments that would most likely yield monoclonal antibodies. After a careful examination of structural features, sequence conservation, hydrophobicity, solvent exposure and numerous other criteria, we designed 12 peptide antigens optimized for mice immunization and the generation of mouse hybridoma clones as previously described [20, 24, 25], (Figure 1 a, b). Six of these peptides are in the N-terminal domain. The remainders are in the C-terminal domain (Figure 1 a, b). Seventeen mouse ascites were produced for initial antibody validation. The hybridomas for these antibodies were then cloned and are stable. The isotype and subclass for each of these antibodies were determined (Figure 1 c). These antibodies provide invaluable reagents to assess Sulf1 levels in tissue and biological fluids by immunohistochemistry and ELISA assay, respectively.

Human Sulf1 (hSulf1) is both a cell surface and secreted protein 4. During cell culture, Sulf proteins are secreted into culture media and can be recovered or enriched from the media. For initial antibody validation, we first generated 293T cells that stably express hSulf1 and prepared condition media (CM) from those cells. Using heparin beads, we pulled down both endogenous and recombinant Sulf1 from CM. The presence of recombinant Sulf1 in the heparin pull-down fraction was confirmed using an anti-His-tag antibody. (Figure 2a). Human SULF1 polyclonal antibody G1.6 (a gift from Dr. Steven D. Rosen, UCSF) [25–27] was used to detect the N-terminal domain of Sulf1 in the fraction (Figure 2a).

Sulf1 shares over 60% amino acid sequence similarity with Sulf2 [5]. To rule out Sulf1 antibodies' cross-reactivity with Sulf2 protein, we generated 293T cells stably expressing

hSulf2. Heparin pull-down assay was used to collect hSulf2 protein from CM derived from H293T hSulf2-expressing cells. The presence of recombinant Sulf2 in the heparin pull-down fraction was confirmed using an anti-His-tag antibody. (Figure 2a). To detect Human Sulf2 monoclonal, we used our previously generated Sulf2 specific monoclonal antibody (8G1) [20, 28] to detect the N-terminal domain of Sulf2 in the heparin-pulled down fraction (Figure 2a).

We screened the seventeen Sulf1 antibodies using the heparin pulled-down hSulf1 and hSulf2 protein fraction. Three out of the seventeen assessed antibodies recognized the rhSulf1. Clone 4-C25 recognized the N-terminal 75 KDa fragment and clone 2-C163, and 5-C29 recognized the C-terminal fragments consistent with their epitopes' location in the Sulf1 sequence (Figure 1). All the three antibodies mentioned above did not cross-react to Sulf2 proteins.

As a secreted protein, Sulf1 is likely to be released in the blood. We tested if these three clones can recognize secreted hSulf1 in plasma samples Clone 4-C25 yielded the strongest Sulf1 signal in plasma (not shown). Figure 3 shows that clone 4-C25 detected Sulf1 in plasma from lung cancer patients, COPD patients, and control healthy individuals (Figure 3). Plasma samples were strongly positive for Sulf1 protein and showed both the 125–130 kDa and 75 kDa bands depicting respectively the unprocessed pro-protein and the amino-terminal subunit of Sulf1 protein [7, 26]. These results demonstrate the utility of these novel antibodies for Sulf1 protein detection by western blot analysis.

Development of SULF1 ELISA

Biological fluids biomarkers are attractive in disease detection because the analysis of body fluids is noninvasive and straightforward [29]. Enzyme-linked immunosorbent assay, ELISA, is one of the commonly used assays to analyze proteomic biomarkers in human body fluids. Unlike Western blot analysis, ELISA requires antibodies that recognize target proteins in their native form.

The establishment of the Sulf1 ELISA assay was technically difficult. A major issue that we had to overcome was optimal combinations for detecting and capturing Sulf1 from Biological fluids. We first screened our 17 antibody clones for Sulf1 detection antibodies using heparin-BSA Capture ELISA assay as previously described [30]. 10% rhSulf1 CM, prepared from the transient transfection of 293T cells, was applied to plastic wells that were coated with heparin-BSA (which binds the SULFs) (Figure 4a). Bound Sulf1 antibodies were detected by sequential reaction with biotinylated anti-mouse secondary antibody and streptavidin-alkaline phosphatase with PNPP as the substrate (405 nm). Remarkably, the most promising Sulf1 detection antibodies we obtained from this heparin-BSA capture ELISA screening were 2-C163, 4-C25, and 5-C29 mAbs. The three Sulf1 mAbs mentioned above are the same antibodies that recognized rhSulf1 in the western blot analysis (Figure 4b). 2-C163, 4-C25, and 5-C29 mAbs were further biotinylated to reduce background signals.

We quantified the amount of Sulf1 in CM from 293T cells that stably express hSulf1 (rhSulf1 CM) (Figure 4b). Notably, these antibodies did not react with hSulf2 protein in CM

from MCF7 human breast cancer line (Figure 4b). We established a linear relationship between the ELISA signal and dilution of rhSulf1 CM (Figure 4c). Among our three Sulf1 biotinylated antibodies, 4-C25 resulted in the best signals and the best R square (>0.99) (Figure 4b and 4c) in heparin-BSA capture ELISA assay using rhSulf1. To further verify the specificity of the heparin-BSA/4-C25 ELISA, we did a Sulf1 depletion assay. rhSulf1 CM was first incubated with protein G beads only or protein G beads conjugated with Sulf1 antibody 4-C25, Sulf2 antibody 8G1, or mouse IgG to deplete Sulf1. We then collected the supernatant after the protein G beads were pelleted and used the depleted supernatant for heparin-BSA/4-C25 ELISA. We only observed Sulf1 signals' depletion when the CM was pre-incubated with the 4-C25 antibody (Figure 4d). The signals were not reduced when the CM was pre-incubated with mouse IgG, 8G1, or protein G beads (Figure 4d). These results indicate that our newly developed heparin-BSA/4-C25 ELISA assay is Sulf1 specific. Furthermore, using purified recombinant human Sulf1 with known concentration, we measured the assay limitation to be 0.86 ng/ml.

Interestingly, when human plasma was analyzed with heparin-BSA/4-C25 ELISA, we observed an inhibition of the signals in human plasma (Figure 5a). The inhibition in signals was dose-dependent as the reduction of signals increased when more plasma was present in the capture step (Figure 5a). Remarkably, we found that pre-incubation of plasma with 4-C25 overcomes this inhibition (Figure 5b and 5c). Furthermore, we showed a linear relationship between the ELISA signal and dilution of rhSulf1 CM (Figure 5d).

In summary, after testing several different strategies, we determined that a Sulf1 sandwich ELISA with heparin-BSA capture of Sulf1 pre-bound to 4–25 mAb followed with an antibody-HRP conjugate detection, was the most sensitive method to detect SULF1 in human plasma. The abovementioned Sulf1 ELISA was then thoroughly validated using a similar strategy as previously described for Sulf2 ELISA assay. We employed conditioned media (CM), with known amounts of SULF1, from HEK293 cell line with stable expression of recombinant SULF1.

Using our new Sulf1 ELISA assay, we analyzed plasma samples from 13 healthy donors, and plasma samples, before surgical resection, from 23 newly diagnosed early-stage NSCLC patients. Our preliminary results reveal that the level of Sulf1 protein is not significantly different in plasma samples from NSCLC patients, as compared with healthy controls. These initial results suggest that Sulf1 plasma levels might not be a useful biomarker for NSCLC early detection. However, these early Sulf1 ELISA assay findings need further validation on a larger clinical cohort with the appropriate age- and gender-matched controls. Nevertheless, these preliminary results provide evidence that Sulf1 protein levels can be quantified in human blood and that our SULF1 ELISA may have utility for such application.

Development of immunohistochemistry protocol for Sulf1 detection in tissues

Our results with the heparin-BSA capture ELISA assay and the rhSulf1 depletion assays showed that 2-C163, 4-C25, and 5-C29 antibodies could recognize the native form of Sulf1 proteins. Next, we sought that determine whether these three antibodies can be used in immunohistochemistry assays for Sulf1 protein detection in tissues. Among these three antibodies, 4-C25 showed strong staining in 293T cells expressing recombinant human Sulf1

(Figure 6a). Furthermore, 4-C25 showed no background in non-transfected cells or cells expressing recombinant human Sulf2 (Figure 6a). Next, we used 4–25 mAb to characterize the expression levels of Sulf1 in tumor sections from NSCLC patients by immunohistochemistry assays. Our results revealed that Sulf1 protein is expressed at higher levels at the invasive front of tumors and lower levels in the surrounding stroma cells (Figure 6b). Also, higher Sulf1 staining was observed in tumor-associated immune cells (Figures 6b and 6c). The results mentioned above are encouraging and suggest that Sulf1 may be involved in NSCLC tumor invasion. Validation of our present findings in a larger clinical cohort may shed light on the Sulf1 role in lung cancer pathogenesis. Our 4-C25 mAb may be a valuable investigative tool in such studies.

DISCUSSION

Using the novel Sulf1 antibodies developed in the laboratory, we tested these antibodies' application on several immunoassays, including Western Blot, ELISA, and IHC. Three of the 17 monoclonal antibodies were able to detect recombinant human Sulf1 using Western blot analysis. Clone 4-C25 recognizes the N-terminal fragment, and clone 2-C163 and 5-C29 recognize the C-terminal fragments. Clone 4-C25 also recognized the native form of Sulf1 in human plasma samples from lung cancer patients, healthy controls, and COPD patients. After screening the 17 clones of antibodies, we developed a new Sulf1 ELISA that can detect recombinant human Sulf1 in simple fluid (e.g., cell culture media). A depletion assay confirmed the specificity of heparin-BSA/4-C25 ELISA toward Sulf1 protein. However, the Sulf1 binding to heparin or the detection antibodies binding to heparin-bound SULF1 was interfered with by other components in human plasma.

Interestingly, the pre-incubation of samples with the detection antibody (i.e., 4-C25) overcame the inhibition from plasma. This modified ELISA protocol allows Sulf1detection in a more complicated fluid environment, such as plasma. The application of those antibodies for immunohistochemistry was also tested. Clone 4-C25 antibody yielded strong Sulf1 staining on the invasive front of tumors from human lung cancer patients. The finding mentioned above implies that the Sulf1 expression associated with tumor invasion and Sulf1 may play an essential role in regulating tumor cell migration.

There is compelling evidence for Sulf1 expression correlation with cancer. SULF1gene upregulation was observed in thirty independent microarray studies comparing tumors and their healthy counterparts in many different cancer types [5]. Although, a tumor suppressor role of Sulf1 has also been proposed [12, 13, 31, 32]. The different observations in Sulf1 expression could be cancer type-dependent or stage-dependent as the expression of Sulf1 is altered during tumor progression and invasion [5, 33–36]. Further understanding of the Sulf1 role in cancer will help elucidate how Sulf1 expression contributes to tumorigenesis. Tools that help characterize and quantify the expression levels of SULF1 in tumor samples biological are much needed to further clarify the Sulf1 role in cancer. The new antibody clone 4-C25 developed in our laboratory has shown promising results in all three immunoassays tested. Other applications, such as Sulf1 immunoprecipitation, could also be performed with this antibody as we have successfully depleted Sulf1 protein from the cell culture media using 4-C25 (Figure 4d).

As a secreted enzyme, Sulf1 is very likely to be released in human blood. Using western blot analysis, we confirmed the Sulf1 protein's presence in human plasma (Figure 3). Significantly, our preliminary results demonstrated the utility of our newly developed Sulf1 ELISA in the detection and quantification of Sulf1 protein levels in human blood from both healthy and disease. In contrast to Sulf1 evaluation by Western blot assay, Sulf1 ELISA allows the analysis of many biospecimens in a relatively short period. The high throughput feature of this ELISA assay makes it a suitable tool for biomarker study. Thus, our Sulf1 ELISA has a potential application as a diagnostic or prognostic tool in diseases with SULF1 dysregulation.

Abbreviations;

CM	conditioned medium
HD	hydrophilic domain
HSPG	heparan sulfate proteoglycan
mAb	monoclonal antibody

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Highlights

- New tools to assess and clarify the SULF1 role in tumorigenesis
- Novel SULF1 mouse monoclonal antibodies suitable for a variety of immunoassays
- SULF1 is present at the invasive front of tumors from NSCLC patients
- Novel SULF1 ELISA to measure its blood levels in healthy and disease
- SULF1 can be detected and quantified in plasma by a novel ELISA assay

(a)

Order	Peptide sequence
1 (N)	PRFRGRIQQERK
2 (C)	RPKNLDVGNKDG
3 (C)	PRDLQASSGGNR
4 (N)	EESSKNIQQSNH
5 (C)	QSYYNKEKGVKK
6 (N)	RESGYRASRSQR
7 (C)	SIHCERELYQSA
8 (N)	RFVHTRQTRSLS
9 (N)	NLYARGFHDKDK
10 (C)	DSSNAVGPPTTV
11 (N)	SVEFEGEIYDIN
12 (C)	KDGGSYDLHRGQ

(b)

MKYSCCALVLAVLGTELLGSLCSTVRSPRFRGRIQQERKNIRPNIILVLTDDQDVELGSLQVMNKTRKIM

EHGGATFINAFVTTPMCCPSRSSMLTGKYVHNHNVYTNNENCSSPSWQAMHEPRTFAVYLNNTGYRTAFF

GKYLNEYNGSYIPPGWREWLGLIKNSRFYNYTVCRNGIKEKHGFDYAKDYFTDLITNESINYFKMSKRMY

PHRPVMMVISHAAPHGPEDSAPQFSKLYPNASQHITPSYNYAPNMDKHWIMQYTGPMLPIHMEFTNILQR

KRLQTLMSVDDSVERLYNMLVETGELENTYIIYTADHGYHIGQFGLVKGKSMPYDFDIRVPFFIRGPSVE

PGSIVPQIVLNIDLAPTILDIAGLDTPPDVDGKSVLKLLDPEKPGNRFRTNKKAKIWRDTFLVERGKFLR

KKEESSKNIQQSNHLPKYERVKELCQQARYQTACEQPGQKWQCIEDTSGKLRIHKCKGPSDLLTVRQSTR

NLYARGFHDKDKECSCRESGYRASRSQRKSQRQFLRNQGTPKYKPRFVHTRQTRSLSVEFEGEIYDINLE

EEEELQVLQPRNIAKRHDEGHKGPRDLQASSGGNRGRMLADSSNAVGPPTTVRVTHKCFILPNDSIHCER

ELYQSARAWKDHKAYIDKEIEALQDKIKNLREVRGHLKRRKPEECSCSKQSYYNKEKGVKKQEKLKSHLH

PFKEAAQEVDSKLQLFKENNRRRKKERKEKRRQRKGEECSLPGLTCFTHDNNHWQTAPFWNLGSFCACTS

SNNNTYWCLRTVNETHNFLFCEFATGFLEYFDMNTDPYQLTNTVHTVERGILNQLHVQLMELRSCQGYKQ

CNPRPKNLDVGNKDGGSYDLHRGQLWDGWEG

(c)

Clone	Isotype	epitope sequence
1-C9	IgG3, Kappa	PRFRGRIQQERK
2-C2	IgG3, Kappa	RPKNLDVGNKDG
2-C4	IgG1, Kappa	RPKNLDVGNKDG
2-C162	IgG2b, Kappa	RPKNLDVGNKDG
2-C163	IgG3, Kappa	RPKNLDVGNKDG
3-C318	IgG2b, Kappa	PRDLQASSGGNR
3-C321	IgG1, Kappa	PRDLQASSGGNR
4-C25	IgG3, Kappa	EESSKNIQQSNH
4-C33	IgG1, Kappa	EESSKNIQQSNH
4-C173	IgG1, Kappa	EESSKNIQQSNH
5-C24	IgG1, Kappa	QSYYNKEKGVKK
5-C29	IgG3, Kappa	QSYYNKEKGVKK
5-C174	IgG1, Kappa	QSYYNKEKGVKK
6-C22	IgG1 Kappa	RESGYRASRSQR
6-C27	IgG3, Kappa	RESGYRASRSQR
7-C150	IgG1, Kappa	SIHCERELYQSA
7-C291	IgG2b, Kappa	SIHCERELYQSA

(d)

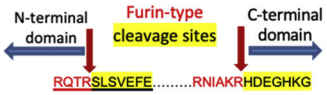

Figure 1. Sequences of peptides used to generate mouse monoclonal antibodies against human Sulf1 protein;

(a) The amino acid sequences of the 12 peptides; (b) The corresponding locations of the 12 peptides in human Sulf1 protein sequence; (c) following immunization of mice with these 12 peptides, 17 hybridomas were generated and the corresponding antibodies were cloned and their isotype and subclass were determined; (d) The Furin-type cleavage sites in Sulf1 that generates the N-terminal and C-terminal domains

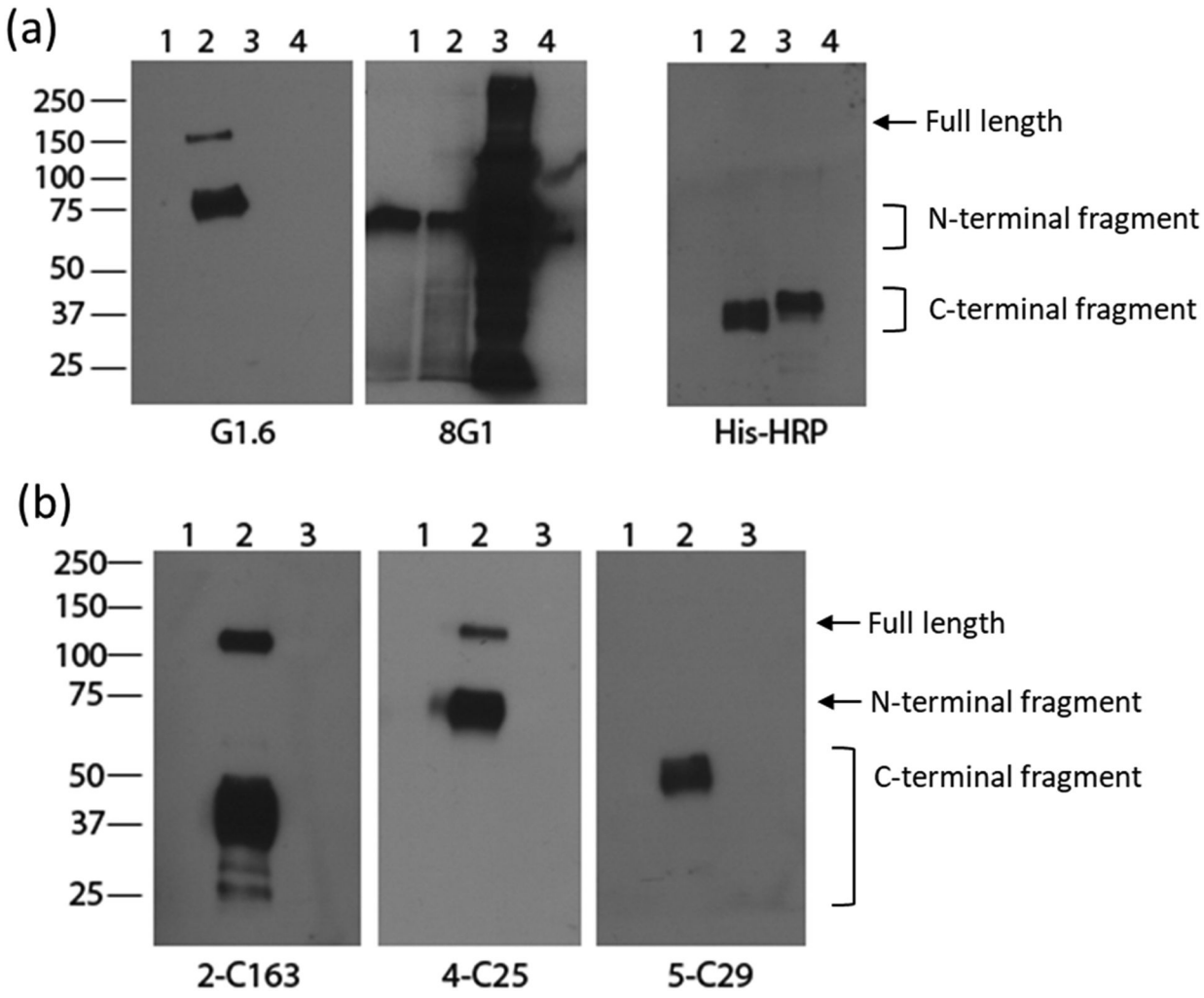

Figure 2. Screen of mouse monoclonal antibodies with recombinant human SULF1.

(a) SULF Western confirmed the expression of recombinant human SULF1 and SULF2 from 293 cells transfected with SULF expression plasmids. 1: CM from 293 cells; 2: CM from 293 cells transfected with SULF1 plasmid; 3: CM from 293 cells transfected with SULF2 plasmid; 4: CM from MCF7 culture. G1.6: anti-SULF1 polyclonal antibody; 8G1: anti-SULF2 monoclonal antibody; His-HRP: anti-His tag, HRP conjugated antibody. (b) Three positive antibody clones that recognized recombinant human SULF1 protein. 1: CM from 293 cells; 2: CM from 293 cells transfected with SULF1 plasmid; 3: CM from 293 cells transfected with SULF2 plasmid.

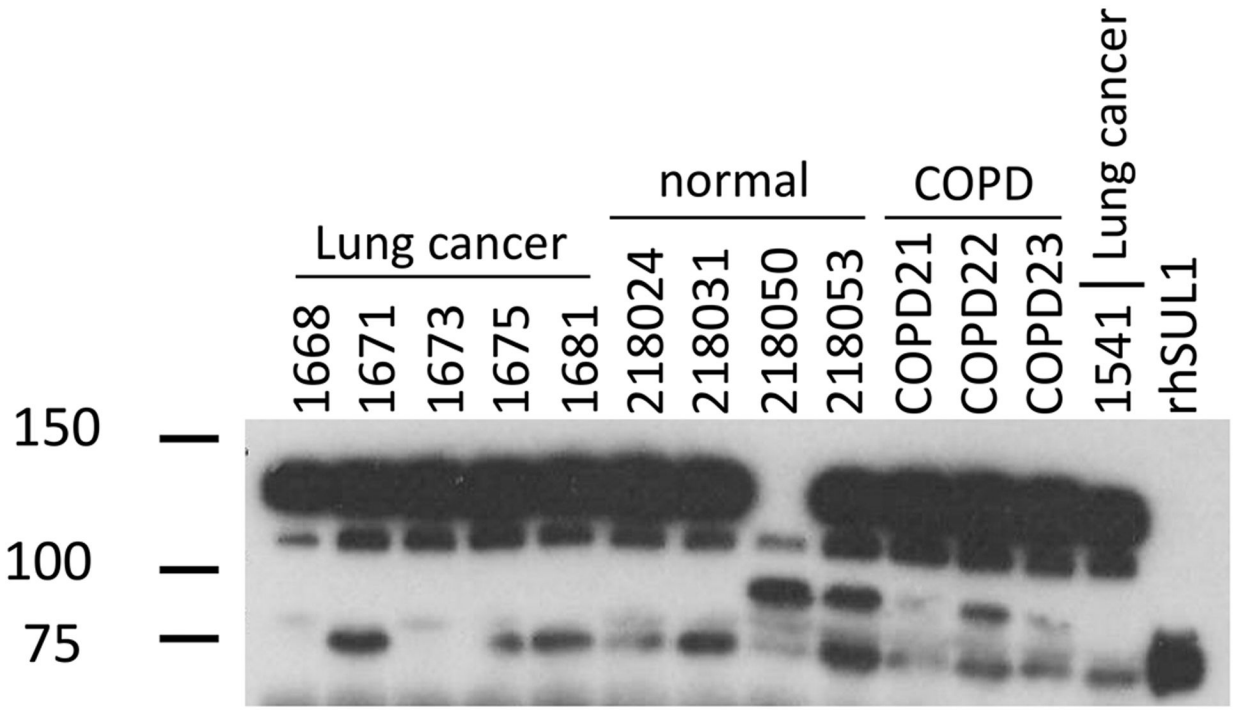

Figure 3. 4-C25 detected SULF1 in human plasma samples from lung cancer patients, normal controls and COPD patients.

0.5 ul of plasma samples were subjected to Western blot analysis using 1 ug/ml 4-C25 antibody. rhSULF1: recombinant human Sulf1 protein.

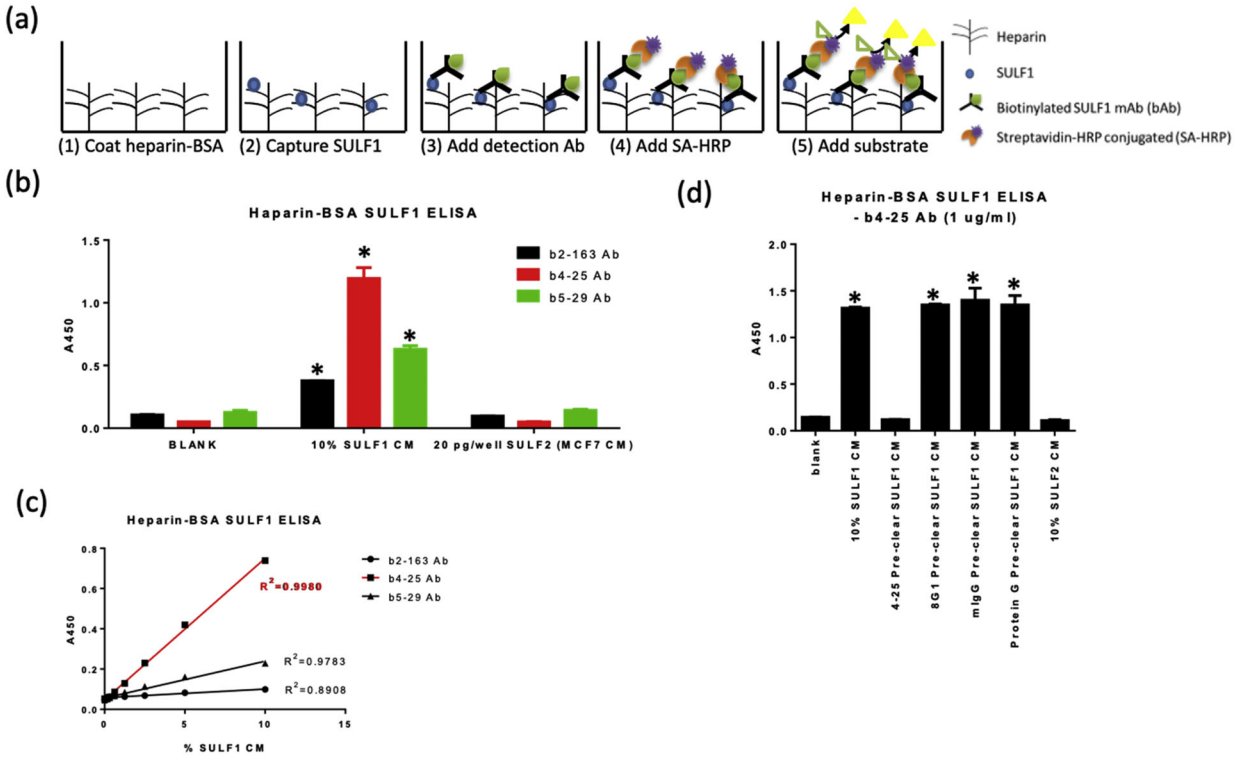

Figure 4. Heparin-BSA SULF1 ELISA.

(a) The steps of heparin-BSA SULF1 ELISA was illustrated. Biotinylated SULF1 mAbs are used as the detection antibody to detect heparin-bound recombinant human SULF1. (b) Heparin-BSA SULF1 ELISA using three different biotinylated SULF1 mAb for detection. All three antibodies recognized SULF1 but not SULF2 protein. (c) The standard curves for heparin-BSA SULF1 ELISA. (d) The ELISA assay is specific to SULF1. Pre-incubation of SULF1 CM with 4-C25, but not 8G1 or mIgG, depleted the ELISA signal. The values shown in graphs are ± SDs for 3 independent determinations (* indicates $p \leq 0.05$ compared to blank).

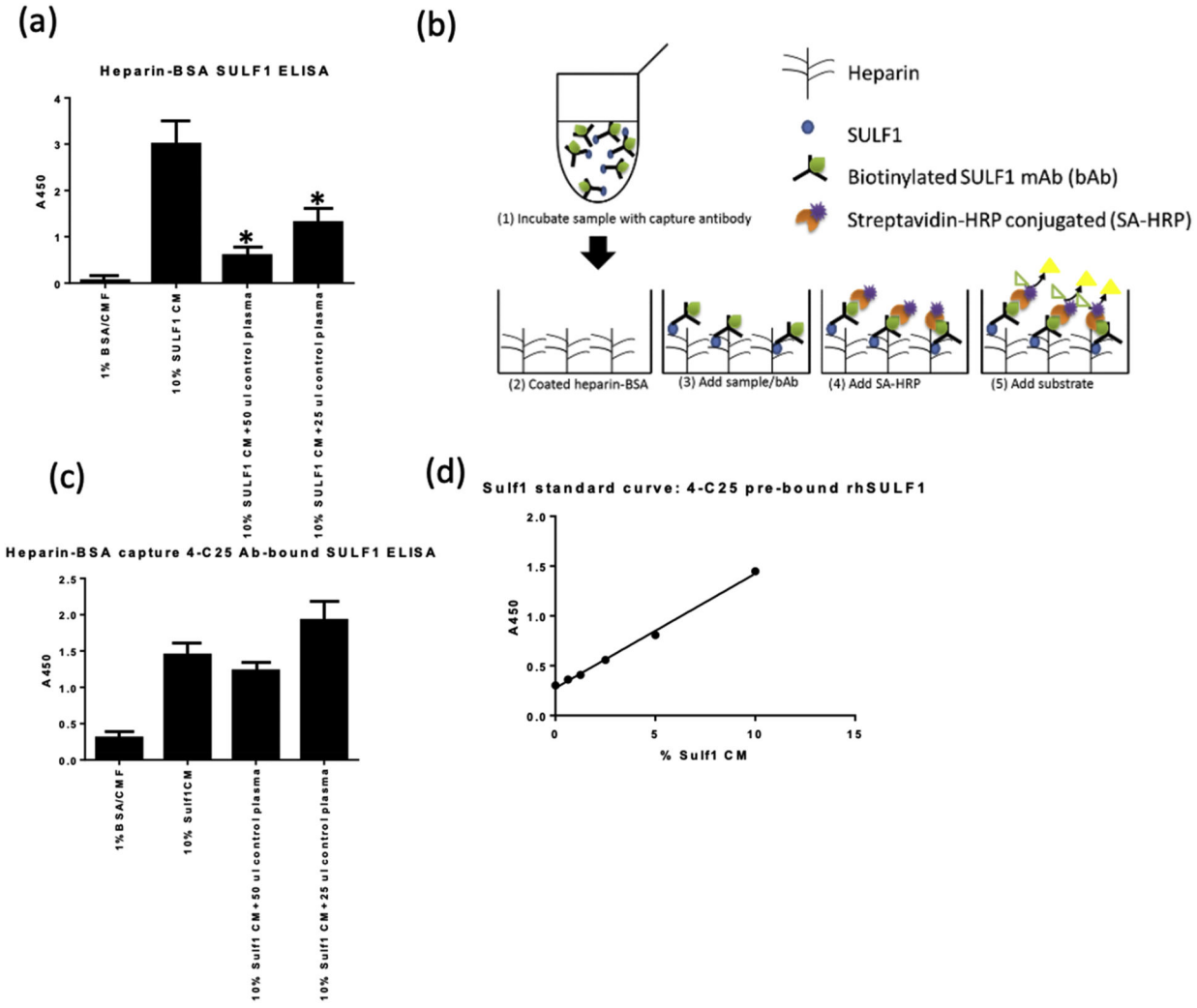

Figure 5. Heparin-BSA capture 4-C25 -bound SULF1 ELISA.

(a) Inhibition of ELISA SULF1 signals in the presence of human plasma using heparin-BSA SULF1 ELISA. (b) Illustration of the modified SULF1 ELISA. Biotinylated 4-C25 is pre-incubated with samples to capture SULF1 before adding to ELISA wells. (c) The presence of human plasma did not interfere with the interaction of 4-C25 to SULF1 protein in the pre-bound ELISA. (d) Standard curve for heparin-BSA capture 4-C25 – bound SULF1 ELISA. The values shown in graphs are ± SDs for 3 independent determinations (* indicates $p \leq 0.05$ compared to 10% Sulf1 CM).

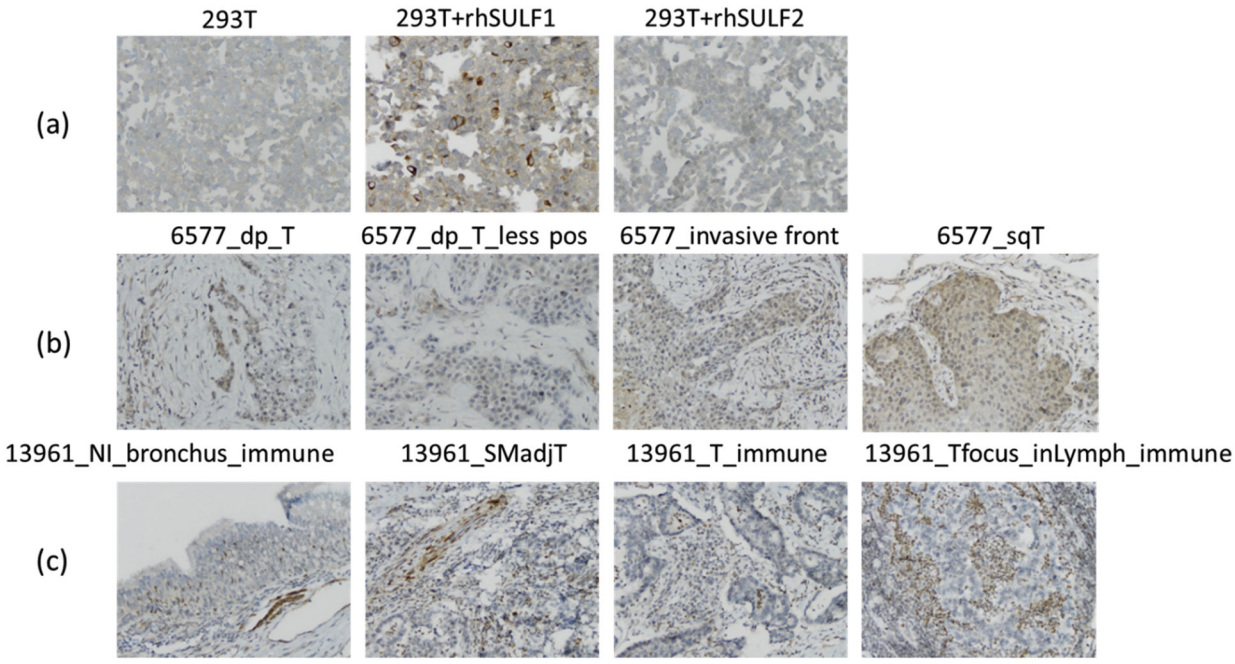

Figure 6. SULF1 immunohistochemistry.

(a) 4-C25 staining of sections from cell blocks prepared using 293T cells, 293T cells expressing recombinant SULF1 or SULF2. 4-C25 showed strong staining in cells expressing SULF1 but not in non-transfected cells or in cells expressing rhSULF2. (b and c) 4-C25 staining of sections from two lung tumors. 4-C25 showed strong staining in immune cells and the tumor invasive front.