Hyaluronic acid present in the tumour microenvironment can negate the pro-apoptotic effect of a recombinant fragment of Human Surfactant Protein-D on breast cancer cells

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Running Title: Innate immune surveillance by SP-D
Abstract

Human surfactant protein D (SP-D) belongs to the family of collectins that is composed of a characteristic amino-terminal collagenous region and a carboxy-terminal C-type lectin domain. Being present at the mucosal surfaces, SP-D acts as a potent innate immune molecule and offers protection against non-self and altered self-such as pathogens, allergens, and tumour. Here, we examined the effect of a recombinant fragment of human SP-D (rfhSP-D) on a range of breast cancer lines. Breast cancer has four molecular subtypes characterised by varied expression of oestrogen (ER), progesterone (PR) and EGF receptors (HER2). The cell viability of HER2 over-expressing (SKBR3) and triple-positive (BT474) breast cancer cell lines (but not of triple-negative cell line (BT20), was reduced following rfhSP-D treatment at 24h. Upregulation of p21/p27 cell cycle inhibitors and p53 phosphorylation (Ser15) in rfhSP-D-treated BT474 and SKBR3 cell lines signified G2/M cell cycle arrest. Cleaved caspase 9 and 3 were detected in rfhSP-D-treated BT474 and SKBR3 cells, suggesting an involvement of intrinsic apoptosis pathway. However, rfhSP-D-induced apoptosis was nullified in the presence of hyaluronic acid whose increased level in breast tumor microenvironment is associated with malignant tumor progression and invasion. rfhSP-D bound to solid-phase HA and promoted tumor cell proliferation. rfhSP-D-treated SKBR3 cells in the presence of hyaluronic acid showed decreased transcriptional levels of p53 when compared to SKBR3 cells treated with rfhSP-D only. Thus, hyaluronic acid appears to negate the anti-tumorigenic properties of rfhSP-D against HER2-over-expressing and triple-positive breast cancer cells.
**Introduction**

The immune-surveillance of transformed cells by innate and adaptive immunity remains one of the targeted area of research for developing therapeutic strategies [1,2]. *In vivo* and *in vitro* studies using cancer models and cell lines have demonstrated compelling involvement of effector immune cells, soluble factors and signalling pathways in anti-tumour immune responses. However, the immune system can also aid in progression of transformed cells by triggering immunosuppression, and promoting angiogenesis, and metastasis of tumour cells [3,4].

Human surfactant protein D (SP-D) is a potent innate immune molecule at pulmonary and non-pulmonary mucosal surfaces [5]. It is a member of collectin family that is involved in the clearance of pathogens and apoptotic/necrotic cells and in modulating inflammatory responses [6]. Like other C-type collectins, SP-D is composed of an N-terminal cysteine rich domain, a triple helical collagenous region, an α-helical coiled neck, and a C-terminal C-type lectin or carbohydrate recognition domain (CRD) [7]. The trimeric CRDs recognise carbohydrate or charged patterns on pathogens and allergens, while collagen region is involved in interaction with receptor molecules present on immune cells in order to trigger clearance mechanisms such as agglutination, enhanced phagocytosis, and oxidative burst [6]. SP-D is primarily synthesised and secreted into the air space of the lungs by alveolar type II and Clara cells, with a key role in surfactant homeostasis by reducing surface tension [6]. However, its extrapulmonary existence is well-established now, ranging from the mucosa of the gastrointestinal and reproductive tracts (including ovaries), and nasal cavity, in the brain and in various exocrine ducts [8,9], conjunctiva, cornea, lacrimal gland, nasolacrimal ducts [8], and synovial fluid [10]. Protective effects of SP-D against a range of pathogens [6,11] and allergens [12–16] are well-documented in the literature. However, recent studies have raised the possibility that SP-D may have an important defence role against tumour.

Direct interaction of SP-D with a number of cancer cells (leukaemia, lung, prostate and pancreatic) has been reported to result in the suppression of cancer progression, migration and invasion, as well as enhanced apoptosis [17–21]. The rfhSP-D treated AML cells were shown to result in cell cycle arrest via activation of G2/M checkpoints, with an increased level of p21 and Try15 phosphorylation of cdc2. rfhSP-D treatment in these AML cells also caused activation of pro-apoptotic markers, such as cleaved caspase 9 and down-regulation of pro-survival protein HMGA1 [21,22]. Recently, exogenous SP-D treatment has been shown to downregulate epidermal growth factor (EGF) signalling by preventing the binding of EGF to EGFR, hence, suppressing the cell proliferation, invasion and migration of A549 human lung adenocarcinoma cells [23]. Recently, rfhSP-D has been shown to induce apoptosis in PS3 mutant (mt) and wild-type (wt) pancreatic adenocarcinoma (PDAC) cell lines (Panc-1p53 mt, MiaPaCa-2p53 mt and Capan-2p53wt), via TNF/Fas-mediated extrinsic pathway [17]. Furthermore, rfhSP-D can also suppress epithelial-mesenchymal transition (EMT) and related gene signatures (Vimentin, Zeb1, and Snail) and cell invasiveness in Panc-1 and MiaPaCa-2 cells via downregulation of TGF-β [24]. In an ovarian cell line, SKOV3, rfhSP-D again triggered apoptosis via Fas-mediated pathway [18]. In both pancreatic and ovarian cancer cell lines, rfhSP-D treatment caused activation of caspase 3 cleavage, induction of pro-apoptotic genes such as Fas and TNF-α. Furthermore, mTOR pathway was also affected by rfhSP-D treatment in both ovarian and pancreatic cancer. rfhSP-D treated SKOV3 cells shown down regulation of Rictor and Raptor mRNA levels, suggesting the possible cause of subsequent inhibition of cell proliferation [17,18]. Additionally, anti-tumour role of rfhSP-D have been reported in androgen resistant and responsive prostate
cancer cells via P53 and pAkt pathways [19]. In a recent bioinformatics study, higher expression of SP-D in ovarian and lung cancer, has been found to be associated with a favourable prognosis [20]. These studies therefore suggest that SP-D has an immune surveillance function against tumour cells. In this context, this study was aimed at investigating the immune surveillance role of SP-D in breast cancer.

Breast cancer is the most common cancer diagnosed in women worldwide contributing to almost 60% mortality rate in lower income countries [25]. There is a large variation in the survival rates worldwide, with an estimated 5-year survival rate of 80% in developed countries and below ~40% in low income-countries [26]. Designing and developing effective treatments are crucial to improve the survival rates and to ensure the best possible quality of life for cancer survivors. After lung cancer, breast carcinomas are the second most leading cause of mortality [27]; ~17.5 million new cases of breast cancer are diagnosed globally, and 8.7 million deaths documented [28]. As per Globocan 2018, approximately 2 million new cases were identified and 6.6% of cancer related deaths was caused by breast cancer. Furthermore, the 5 year prevalence rate of breast cancer was found to be approximately 6.8 million [25]. Breast cancer is characterized by an abnormal growth of malignant cells in the mammary glands. The physiological conditions that lead to breast tumorigenesis include inherited genetic mutation and epigenetic modifications, which can lead to premalignant transformation of mammary cells [29]. The development of advanced breast tumour is a consequence of immune selection and immune evasion [30]. Breast cancer is subdivided into different molecular subtypes; luminal A, luminal B, triple negative, human epidermal growth factor receptor (HER) 2 enriched, basal and normal-like tumours [31,32]. The development and metastasis of cancer, including breast cancer, appears to be influenced by innate immune surveillance molecules and associated inflammatory mediators in the tumour environment [33,34].

Here, we examined possible protective effects of rfhSP-D in triple-negative (ER−/PR−/HER2−), triple-positive (ER+/PR+/HER2+) and HER2+ overexpressing breast cancer cell lines. We also examined a possible interaction between SP-D and HA, the most abundant component of extracellular matrix (ECM), which plays an important role in inflammation, angiogenesis, fibrosis, and cancer progression [35]. Interaction of breast cancer cells with HA can sustain tumour growth and promote malignant progression. Altered synthesis of HA in early and late stage of ductal breast carcinoma in situ (DCIS) microenvironment is correlated with tumour stage progression and invasion [36]. Several studies provide strong evidence that HA participates in the regulation of breast tumour cell migration and invasion in vitro, and tumour growth and progression in vivo [37–39]. We report, for the first time, that rfhSP-D interacts with HA that negates its anti-tumour properties i.e. in the presence of HA, rfhSP-D is unable to induce apoptosis in triple-positive and HER2 overexpressing breast cancer cell lines. Therefore, neutralizing the negative effect of HA on rfhSP-D can have crucial implications for the development of therapeutic strategies.

Materials and Methods

Expression and Purification of rfhSP-D

Expression and purification of rfhSP-D was performed as published earlier [19]. Briefly, Escherichia coli BL21 (ADE3) pLysS bacterial strain (Invitrogen) was transformed with plasmid pUK-D1, composed of cDNA sequences for α-helical neck and CRD region of human SP-D. 25 ml bacterial primary inoculum was inoculated into 500 ml of lysogeny broth medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol (Sigma-Aldrich), and grown to OD600 of 0.6. The grown bacterial cells were then induced with 0.5 mM
isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich) for 3 h. The IPTG induced bacterial cell pellet was treated with lysis buffer (50 mM Tris–HCl pH7.5, 200 mM NaCl, 5 mM EDTA pH 8.0, 0.1% v/v Triton X-100, 0.1 mM phenyl-methyl-sulfonyl fluoride, 50 µg/ml lysozyme) and followed by sonication (five cycles, 30 s each). The sonicated bacterial cell pellet was centrifuged at 12,000 × g for 30 min, followed by denaturation and renaturation of rhfSP-D inclusion bodies using refolding buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 10 mM 2-Mercaptoethanol) containing 8 M urea. The refolded dialysate was then loaded onto a maltose-agarose affinity column (5 ml; Sigma-Aldrich) and rhfSP-D was eluted using 10mM EDTA buffer containing 50 mM Tris–HCl, pH 7.5 and 100 mM NaCl. Eluted rhfSP-D fractions were then tested for endotoxin levels using QCL-1000 Limulus amoebocyte lysate system (Lonza) (Sigma-Aldrich). The endotoxin levels were found to be found to be ~5 pg/µg of rhfSP-D, and purity of proteins was analysed via 15% w/v SDS-PAGE, and its immunoreactivity was determined via western blotting.

Cell culture and Treatments

Human breast cancer cell lines, triple-negative BT20 (ER-/PR-/HER2-) (ATCC-HTB19), triple-positive BT474 (ER+/PR+/HER2+) (ATCC-HTB20) and HER2-positive SKBR3 (ER-/PR-/HER2+) (ATCC-HTB30), were cultured in complete RPMI medium (Sigma-Aldrich), supplemented with 10% v/v Fetal Bovine Serum (FBS), 2mM L-glutamine, 100U/ml penicillin (Sigma-Aldrich), 100μg/ml streptomycin (Sigma-Aldrich) and 1mM sodium pyruvate (Sigma-Aldrich), and left to grow at 37°C under 5% v/v CO₂. Since these cell lines were adherent, cells were detached using 2 × Trypsin-EDTA (0.5%) (Fisher Scientific) for 10 minutes at 37°C, then centrifuged at 1500 rpm for 5 minutes, and re-suspended in complete RPMI. Cell number and viability was assessed by mixing an equal volume of the cell suspension and Trypan Blue (0.4% w/v) (Fisher Scientific) and counting using a haemocytometer with Neubauer chamber (Sigma-Aldrich).

Fluorescence microscopy

BT20, BT474 and SKBR3 (50,000) cells were grown on coverslips and incubated with rhfSP-D (10 µg/ml) in serum free RPMI medium for 1 h for analysing cell binding, and 24 h for apoptosis induction. For binding experiments, the coverslips were washed three times with PBS, and incubated with polyclonal anti-rabbit human SP-D antibody (1:200) (Medical Research Council Immunochemistry Unit, Oxford) for 1 h at room temperature. Following washes with PBS, the cells were incubated with goat anti-rabbit IgG conjugated to Alexa Fluor® 488 (1:200) (Abcam) and Hoechst (1:10,000) (Sigma-Aldrich) for immunofluorescence analysis. For apoptosis induction analysis, the cells were incubated with FITC Annexin V (1:200) and PI (1:200) diluted in Annexin V-binding buffer for 15 min at RT in dark. After PBS wash three times, the coverslips were mounted on slides and viewed under a HF14 Leica DM4000 microscope.

Flow cytometry

BT20, BT474 and SKBR3 (0.4 × 10⁶) cells were grown in a 6 well plate and incubated with rhfSP-D (20 µg/ml), along with an untreated, for 24 h. The cells were then detached using 2 × Trypsin-EDTA (0.5%) (Fisher Scientific) and centrifuged at 1,500 × g for 5 min. Staurosporine (1µM/ml) was used as a positive control of apoptosis. Following washes with PBS, the cells were incubated for 15 mins with goat anti-rabbit IgG conjugated to Alexa Fluor® 488 (1:200) (Abcam) and Hoechst (1:10,000) (Sigma-Aldrich) for apoptosis analysis. After washing with PBS three times, apoptosis was measured using Novocyte Flow
Cytometer. Compensation parameters were acquired using unstained, untreated FITC stained, and untreated PI stained for all three cell lines. For solid phase studies, 6 well plates were coated with HA (20 μg/ml) (a kind gift from Prof. Ivan Donati, Department of Life Sciences, University of Trieste) overnight at 4°C with and without rfhSP-D (20 μg/ml). The wells were washed with PBS and 0.4 × 10^6 cells were added to the HA/rfhSP-D-coated wells and incubated 37°C for 24h. For proliferative studies, the cells were washed with PBS, and incubated with anti-mouse Ki-67 (Bio-Legend) diluted in permeabilization reagent of the FIX&PERM kit (Fisher Scientific) for 30 minutes at room temperature. After PBS washes, the cells were probed with goat anti-mouse-FITC conjugate (1:200) (Fisher Scientific) for 30 minutes at RT in dark. Cells (12,000) were acquired for each experiment and compensated before plotting the acquired data.

Purification of full-length SP-D from Breast cancer cell lines

BT20, BT474 and SKBR3 (0.4 × 10^6) culture medium was collected and centrifuged at 5000 RPM for 10 minutes. The centrifuged supernatants were passed through a maltose agarose-affinity column. The bound full-length SP-D was elutes using elution buffer, containing 50 mM Tris–HCl, pH 7.5, 100 mM NaCl and 10mM EDTA. 1 μg/ml full-length SP-D protein yield was purified from 100 ml of total culture medium. The purity of eluted fractions (10 μg/ml) was analysed via 15% w/v SDS-PAGE, and its immunoreactivity was determined via western blotting. For western blotting analysis, the eluted full-length SP-D was heated for 10 minutes at 100°C and subjected to SDS-PAGE (12% v/v). The proteins were then electrophoretically transferred onto a nitrocellulose membrane (320mA for 2h) in 1× transfer buffer (25mM Tris–HCl pH 7.5, 190 mM glycine, and 20% methanol). The membrane was blocked using 5% w/v dried milk powder (Sigma-Aldrich) in PBS overnight at 4°C, followed by washing with PBS three times 5 mins each. The membrane was then incubated with polyclonal anti-rabbit human SP-D antibody (MRC immunohistochemistry Unit, Oxford) (1:1000), followed by probing with Protein-A-HRP (Sigma-Aldrich) (1:1000). Following washing with PBST, the membrane was developed using 3′-diaminobenzidine substrate kit.

Apoptosis analysis of breast cancer cells treated with BT474-derived full-length SP-D

After western blotting confirmation, the purified full-length SP-D from breast cancer culture was subjected to apoptosis assay using Annexin V/FITC kit (Bio-legend). BT20, BT474 and SKBR3 cells (0.4 × 10^6) were incubated with 20 μg/ml of culture purified SP-D and incubated for 24 h. The cells were then detached using 2 × Trypsin-EDTA (0.5%) (Fisher Scientific) and centrifuged at 1,500 × g for 5 mins. Following washes, the cells were then incubated with both FITC and PI dye (1:200) for 15 minutes at dark. After washing with PBS, the cells were subjected to flow cytometric analysis. Compensation parameters were acquired using unstained, untreated FITC stained, and untreated PI stained for all three cell lines.

Immunohistochemical analysis

Surgical breast cancer tissues and adjacent peritumoral mammary parenchymas were selected following ethical approval by the University Hospital of Palermo Ethical Review Board (approval number 09/2018). TNBC, HER2+, Luminal B and Luminal A breast cancer tissues were used, while normal breast tissue was used as a control. Immunohistochemistry (IHC) was performed using a polymer detection method. Briefly, tissue samples were fixed in 10% v/v buffered formalin and paraffin embedded. 4 μm-thick tissue sections were deparaffinised and rehydrated. The antigen unmasking technique was carried out using
Novocastra Epitope Retrieval Solutions, pH 6 EDTA-based (Leica Biosystems) in thermostatic bath at 98°C for 30 min. Sections were brought to RT and washed in PBS. After neutralization of the endogenous peroxidase with 3% v/v H$_2$O$_2$ and Fc blocking (Novocastra, Leica Biosystems), the samples were incubated overnight at 4°C with mouse anti-human SP-D monoclonal antibody (1:800) (Abcam). Staining was revealed via polymer detection kit (Novocastra, Leica Biosystems) and AEC (3-amino-9-ethylcarbazole, Dako, Denmark) substrate chromogen. Slides were counterstained with Harris Hematoxylin (Novocastra, Leica Biosystems). Sections were analysed under the Axio Scope A1 optical microscope (Zeiss); microphotographs were collected through the Axiocam 503 colour digital camera (Zeiss) using the Zen2 software.

Alcian blue staining was carried out to detect HA in the breast cancer tissue sections. After deparaffinization and rehydration, tissue sections were incubated with a solution of 1% Alcian blue (Dako) dissolved in 3% acetic acid, pH 2.5, for 30 min at RT. After washing them under running water for 10 min, the sections were dehydrated in progressively increasing percentages of ethanol and mounted. Slides were examined under a Leica DM 3000 optical microscope; images were acquired using a Leica DFC320 digital camera (Leica Microsystems, Wetzlar, Germany).

**Adhesion Assay**

BT20, BT474 and SKBR3 (0.5 × 10$^5$) cells were labelled with FAST Dil fluorescent dye (Molecular Probes, Invitrogen) and allowed to adhere to 96 microtiter wells pre-coated with 20 μg/ml of HA, rfhSP-D and BSA. The adhesion of cells was measured after 35 minutes of incubation at 37°C under 5% CO$_2$. The non-adhered cells were washed off with PBS, and the remaining cells were lysed using 10 mM Tris–HCl, pH 7.4 + 0.1% v/v SDS. The plate was read at 544nm using Infinite200 (TECAN). Results were expressed as adhesion percentage with reference to a standard curve established using an increasing number of FAST Dil labelled cells.

**Intracellular signalling analysis**

Signalling pathway was analysed using the ‘PathScan Intracellular Signalling Array Kit (Cell Signalling Technology). Briefly, 0.5 × 10$^6$ breast cancer cells lines were grown in a 6 well plate in serum free RPMI medium. Cells were then left to adhere to HA and rfhSP-D (20 μg/ml) pre-coated plates, and incubated at 37°C for 25 min. The unbound cells were then washed with cold PBS and lysed in ice-cold cell lysis buffer containing a cocktail of protease inhibitors (Roche Diagnostics). The Array Diluent Blocking buffer was added to each well on the multi-well gasket, and RT for 15 minutes. After decanting the Array Blocking Buffer, 75 μl of totally lysate (0.8 mg/ml) was added to each well and incubated for 2 h RT on an orbital shaker. The well contents were decanted and washed with 100 μl of 1x Array Wash Buffer three times (5 minutes each wash). The wells were then incubated with biotinylated detection cocktail antibody for 1 h at RT, followed by incubation with Streptavidin-conjugated DyLight 680 for 30 minutes. The fluorescence readout was measured via LI-COR Biosciences Infrared Odyssey imaging system (Millennium science) and data was processed by the software Image studio 5.0.

**Western Blotting**

BT20, BT474 and SKBR3 (0.4 × 10$^6$) cells were seeded in a 6 well plate and treated with rfhSP-D (20 μg/ml) for 24h, along with an untreated control, in a serum-free RPMI medium. After removing the medium, the cells were lysed using a lysis buffer (50 mM Tris-HCl pH 6.8,
2% v/v SDS, 2% v/v β-mercaptoethanol, 10% v/v glycerol and 0.1% w/v bromophenol blue). The lysed cells were then sonicated for 15s and the sonicated samples were heated for 10 minutes at 100°C and subjected to SDS-PAGE (12% v/v). The proteins were then electrophoretically transferred onto a nitrocellulose membrane (320mA for 2h) in 1× transfer buffer (25mM Tris–HCl pH 7.5, 190 mM glycine, and 20% v/v methanol). The membrane was blocked using 5% w/v dried milk powder (Sigma-Aldrich) in PBS overnight at 4°C, followed by washing with PBS three times (5 min each). For apoptosis studies, the membrane was incubated with rabbit anti-human caspase primary antibodies (anti-cleaved caspase 9 and anti-cleaved caspase 3; Cell Signalling) at RT for 1 h. The membrane was washed with PBST (PBS+ 0.05% Tween 20) three times, 10 minutes each, followed by incubation with secondary Goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugate (1:1000; Fisher Scientific) for 1 h at RT. Following washes with PBST, the membrane was developed using 3′-diaminobenzidine (DAB) substrate kit (Thermo Fisher).

**ELISA**

For HA (1500kDa) binding analysis, a constant concentration of HA (20 μg/ml) was coated overnight at 4°C using carbonate/bicarbonate (CBC), pH 9.6 buffer, followed by blocking with 2% w/v BSA at 37°C. A recombinant maltose binding protein (MBP) was used as a negative control in this experiment. After three washes with PBST, rfhSP-D (5, 10, or 20 μg/ml) was incubated in buffer containing 5 mM CaCl$_2$ for 2h at 37°C. After washing with PBST three times, the binding was assessed by polyclonal anti-rabbit human SP-D antibody (1:5000) and incubated at 37°C for 1h. The wells were washed again with PBST three times, and incubated with protein A-HRP secondary conjugate (1:5000) (Sigma-Aldrich) for 1h at 37°C. After washes with PBST, the binding was detected using 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich). The reaction was stopped using 2N H$_2$SO$_4$ and the absorbance was read at 450nm using iMark™ microplate absorbance reader (Bio-Rad).

**Quantitative RT-PCR**

BT20, BT474 and SKBR3 (0.4 × 10$^6$) cells were added on pre-coated 6 well plate with HA (20 μg/ml), HA+ rfhSP-D (20 μg/ml), and rfhSP-D (20 μg/ml), and incubated at 37 °C for various time points. The cell pellet for each time point was subjected to RNA extraction using GenElute Mammalian Total RNA Purification Kit (Sigma-Aldrich). RNA samples were then treated with DNase 1 (Sigma-Aldrich), the total RNA concentration and purity was determined using 260:280 nm ratio using NanoDrop 2000/2000c (Thermo-Fisher Scientific). 2 μg of total RNA was used for cDNA synthesis using High Capacity RNA to cDNA kit (Applied Biosystems). Primer sequences used in this study were designed for specificity using the Primer-BLAST software (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). p21 and p27 mRNA levels were estimated by quantitative RT-PCR using the following primers: 5′-TGGAGACTCTCAGGGTCGAAA-3′ and 5′- CGCGTTTTGGAGTGATAGAA-3′ for p21 and 5′-CCGGTGGACCACGAAGAGT-3′ and 5′-GCTCGCCTCTCCTCATGTCTC-3′ for p27. Briefly, the qPCR reaction consisted of 5 μl iQ SYBR Green Supermix (Bio-Rad, Milan, Italy), 75 nM of forward and reverse primer and 500 ng template cDNA in a 10 μl final reaction volume. qRT-PCR was performed on a Rotor-Gene 6000 (Corbett, Experla, Ancona, Italy). The melting curve of the reactions were recorded between 55 °C and 99 °C with a hold every 2s. The Comparative Quantification (CQ) method was used to determine the relative amount of gene expression in each sample, and the data analysed using Rotor Gene 1.7 software (Corbett Research) [40]. The CQ method is specific.
in calculating the efficiencies of each gene for each individual PCR reaction and is based on the second differential maximum method. The samples were normalized using the expression of human TATA binding protein (TBP) rRNA. Assays were conducted in triplicates.

**Statistical Analysis**

Graphs were generated using GraphPad Prism 6.0; and the statistical analysis was carried out using unpaired one-way ANOVA test. Error bars represents SD or SEM (n=3), as indicated in the figure legends. The significant values were measured based on *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 between treated and untreated samples.

**Results**

A recombinant form of truncated human SP-D binds breast cancer cell lines

rfhSP-D comprising the 8 Gly-X-Y repeats, neck and CRD regions of human SP-D was affinity-purified and migrated at ~20kDa on SDS-PAGE under reducing conditions (Figure 1A). The immunoreactivity of purified rfhSP-D was confirmed via western blotting using polyclonal anti-human SP-D antibody raised in rabbit against native human SP-D purified from lung lavage (Figure 1B). BSA was used as a negative control protein. Purified rfhSP-D (5 μg/ml) bound breast cancer cell lines, triple negative- BT20 (ER−/PR−/HER2−), triple-positive BT474 (ER+/PR+/HER2+), and HER2-positive SKBR3 (ER−/PR−/HER2+) (Figure 1D). The nucleus of the cell was stained with Hoechst dye, while the membrane localisation of the bound rfhSP-D was revealed using green FITC/anti-human SP-D conjugate. All rfhSP-D treated breast cancer cell lines showed a similar ‘cluster’ like binding pattern on the cell membrane (Figure 1D). No FITC was detected in the un-treated controls (Figure 1C), probed with both primary and secondary antibodies, suggesting the specificity of rfhSP-D binding.

Apoptosis induction by rfhSP-D in breast cancer cell lines

The quantitative and qualitative analysis of apoptosis induction by rfhSP-D was performed using flow cytometry (Figure 2A, B) and immunofluorescence microscopy (Figure 2C, D). A significant proportion of BT474 and SKBR3 breast cancer cells treated with rfhSP-D (20 μg/ml), underwent apoptosis at 24h as revealed by FACS analysis (Table 1). BT474 (~61%) and SKBR3 (~68%) cell lines were more susceptible to apoptosis induction when immobilised rfhSP-D (20 μg/ml) (Figure 2A) was used; there was no effect on BT20 cell line. However, rfhSP-D in solution induced apoptosis in BT474 (~34%), SKBR3 (~53%) (Figure 2B), again rfhSP-D had no significant effect on BT20 cell line (Figure 2A) (Table 1). The integrity of the cell membrane was intact in untreated cells, and hence, the cells were still viable, blocking the translation of PS from inner cell membrane to outer plasma membrane, and preventing the PS - Annexin V interaction. Nearly 56% BT474 and SKBR3 cells were both stained FITC and propidium iodide (PI) positive, suggesting that annexin V/FITC was able to bind to phosphatidylserine (PS) found on the cell surface of the cells undergoing apoptosis. However, BT474 cells stained for PI alone were more compared to SKBR3 cell line, suggesting that BT474 cells were either at the late stage of apoptosis or undergoing necrosis. The percentage of viable cells in the untreated (cells only) sample was significantly higher as compared to treated sample; BT474 (~92%), and SKBR3 (~96%), suggesting that apoptosis induction was rfhSP-D protein-specific mediated effect (Figure 2A). Staurosporine (1μM/ml) was used as a positive control for apoptosis studies. Staurosporine treated BT20, BT474 and SKBR3 showed approximately ~70% of apoptosis induction at 24h (Figure 2A&B). Staurosporine is a well-known therapeutic potent apoptosis inductor, which is suggested to
inhibit tumour cell growth and proliferation by triggering cell death via intrinsic apoptotic pathway [41]. Fluorescence microscopy analysis in BT474 and SKBR3 cell lines treated with rhfSP-D revealed a positive staining for cell membrane integrity marker, Annexin V (conjugated to FITC), and disoriented cell membrane morphology (Figure 2C&D). Thus, PI positive staining was seen only in treated cell lines (Figure 2D) compared to un-treated cells only controls (Figure 2C), indicating no occurrence of late apoptotic or necrosis. Whereas, no positive staining of Annexin V or PI was detected in BT20 treated cells and were similar to the untreated control.

**rfhSP-D induces apoptosis in BT474 and SKBR3 cell lines via intrinsic pathway**

Since rhSP-D caused apoptosis in BT474 and SKBR3 cells, we further examined the likely apoptotic pathway being triggered following rhfSP-D treatment. Apoptosis can be initiated through intrinsic or extrinsic pathways. Expression of caspases were tested in rhfSP-D treated breast cancer cells lines. Western blotting analysis of rhfSP-D treated BT474 and SKBR3 cells at 24h revealed cleavage of caspase 9 (~37kDa) and 3 (~17kDa), suggesting the trigger of intrinsic apoptosis pathway (Figure 3). As expected, cleavage of caspase 3 (Figure 3A) and 9 (Figure 3B) was not detected in BT20 and untreated (cells only) controls. Furthermore, cleaved caspase 8 was tested as a marker for extrinsic pathway, but no difference was observed between rhfSP-D treated cell lines and untreated controls (Data not shown).

**Expression of human SP-D and HA in Breast cancer tissues**

The expression of human SP-D was analysed in 8 cases of invasive ductal carcinoma, No Special Type (NSP), comprising two cases for each molecular class (Luminal-A, Luminal-B, Her2-Neu and Triple Negative) based on the expression of the estrogen receptor (ER), the progesterone receptor (PR) and the human epidermal growth factor receptor 2 (Her2-Neu) status. Among biological variables considered, beside to the different molecular class, the neoplastic tissues presented a different grade tumor, computed with the Nottingham Score, varying from moderate (G2) to undifferentiated (G3). Indeed, the molecular classification allows to identify four different classes based on the expression of the estrogen and progesterone receptor (ER and PR) and the human epidermal growth factor receptor 2 (Her2-Neu) status and called Luminal-A, Luminal-B, Her2-Neu and Triple Negative. The molecular subtypes reflect the neoplastic heterogeneity of breast cancer and differ in gene expression patterns, clinical and morphological features, response to treatment and outcome. Our aim was to evaluate the presence and distribution of SP-D in all molecular subtypes of breast cancer to dissect its biological role in neoplastic progression and to consider a potential predictive and/or prognostic marker of mammary carcinoma. Our data appears to suggest a heterogeneous inter and intra-tumor expression of SP-D within the molecular subtypes. In this study, immunohistochemical (IHC) staining for SP-D highlighted its cytoplasmic expression both in the neoplastic tissue and the healthy peritumoral mammary parenchyma, as previously demonstrated [20]. Indeed, SP-D presence was evident in cytoplasmic labelling and was highly expressed by the ductal epithelium of peritumoral mammary parenchyma and the neoplastic sub-clones of luminal A type, while its reduced expression was evident in triple-negative breast cancer (TBNC) (Figure 4A). As regarding the Luminal B and HER2 groups, showed a heterogeneous expression of rhfSP-D (Figure 4A&B). We also examined serial sections and the localization of HA within the tumour microenvironment following histochemical analysis of normal and breast cancer tissue specimens. As shown in Figure 4A, Alcian blue staining highlighted the presence of
acid mucins, containing HA and sialic acid [42–44], in the tumour stroma, around the
tumour cells and was very faint in peritumoral mammary parenchyma in all the molecular
classes of breast cancer considered. The luminal B and Her2 specimens were characterized
by a variable SP-D expression within the tumour. In this case the luminal B and HER2 groups,
Alcian blue staining also showed a greatly heterogeneous expression of HA. Thus, a stronger
staining for Alcian blue was evident where a lower presence of SP-D was detected,
suggesting that a modification of extracellular matrix might be associated with a variable
distribution of SP-D (Figure 4A; G-L).

rfhSP-D binds to high molecular weight HA

Confirmation of the presence of HA in the breast cancer tissues prompted us to examine if
rfhSP-D binds to HA and can modulate the nature of rfhSP-D-breast cancer cell interaction.
In direct binding ELISA, rfhSP-D bound to solid phase high molecular (1500 kDa) HA in a
dose-dependent manner (Figure 5A). Immobilised rfhSP-D (20 μg/ml) alone as well as HA
(20 μg/ml)-bound rfhSP-D were then allowed to interact with breast cancer cell lines. Cell
adhesion assay was performed by labelling the cells with the fluorescent probe FAST Dil
(Figure 5B). All three breast cancer cell lines (BT20, BT474, and SKBR3) were able to adhere
to HA, rfhSP-D and to HA-bound rfhSP-D. rfhSP-D enticed a greater cell adhesion when
compared to HA alone or HA-bound-rfhSP-D; all three cell lines bound to similar extents
(Figure 5B).

HA binding modulates the ability of rfhSP-D to induce apoptosis

The implication of HA-rfhSP-D interaction on apoptosis induction in breast cancer cells was
investigated (Figure 6A). Breast cancer cells incubated on pre-coated HA, HA + rfhSP-D and
rfhSP-D alone wells, along with untreated (cells only) cells, were stained after 24h with
Annexin V/FITC; the quantification of apoptosis was carried out via using flow cytometry
(Figure 6A). Addition of HA to rfhSP-D reduced apoptosis induction by ~45%. Since we
noticed a reduced apoptosis induction by HA+ rfhSP-D, we considered if rfhSP-D induced a
proliferative response in combination with HA. Thus, HA +rfhSP-D treated breast cancer
cells were stained with anti-mouse Ki-67 antibody to detect the percentage proliferation
(Figure 6B). Ki-67 is a well-known nuclear protein associated with active cell proliferation
and is expressed throughout the active cell cycle, including G1, S, G2 and M phases. Addition
of HA in rfhSP-D treated SKBR3 cells resulted in approximately ~30% cell proliferation
(Figure 6B), suggesting that HA negated pro-apoptotic effects of rfhSP-D in breast cancer
cell lines. In the case of BT474, only ~47% of proliferative cells were seen among rfhSP-D
treated BT474 cells, while HA + rfhSP-D treated BT474 cells showed a higher proliferation
(~95%) (Figure 6B). HA alone treated BT474 cells showed ~88% of Ki-67 positive cells, while
~66% of proliferative cells were seen in HA treated SKBR3 cells. However, proliferation of
BT20 cells was not affected by rfhSP-D or HA treatment, suggesting that these cells continue
to grow. These percentage proliferations are compared to untreated control (cells only).
Given the presence of SP-D in the breast cancer tissues, we made an attempt to purify SP-
D from the culture supernatants of the breast cancer cells on the assumption that breast
cancer cells can also be a likely source of SP-D in the tumour microenvironment. We could
purify SP-D only from BT474 cell culture (Figure 6C). However, the purified SP-D was not
able to induce apoptosis in BT474 cells (Figure 6D).
Activated p53 is a known mediator of DNA damage, oxidative burst, cell cycle arrest and induction of apoptosis. Therefore, this study aimed at analysing the levels of activated p53 in rfhSP-D treated breast cancer cells. Increased levels of p53 phosphorylation at Ser15 was detected in rfhSP-D treated SKBR3 cells (Figure 7A), while down-regulation was seen in BT20 cell line (Figure 7A), suggesting that p53 upregulation may also have contributed to p53 dependent apoptosis induction in SKBR3 cells. In addition, increased transcript levels of p21 and p27 were observed in rfhSP-D treated BT474 and SKBR3 cell lines (Figure 7B) at 12h time point, suggesting the possibility of Cdc2-cyclin B1 reduction leading to G2/M cell cycle arrest. Reduced levels of phosphorylated p53, caspase 3, p21 and p27 was observed in HA+ rfhSP-D treated SKBR3 and BT474 cells (Figure 7A & B), suggesting the reduced apoptosis induction by addition of HA. In the case of BT20, no significance difference was found in terms of p21 and p27 mRNA expression levels between rfhSP-D and HA + rfhSP-D treated BT20 cells.

**Discussion**

In this study, we show that rfhSP-D binds to all breast cancer cell lines tested; BT20 (ER+/PR−/HER2−), BT474 (ER+/PR+/HER2+) and SKBR3 (ER+/PR+/HER2+). However, the receptor SP-D is binding to on the cancer cell surface is not yet known. Exogenous treatment with rfhSP-D reduced cell viability of BT474 and SKBR3 cell lines, and induced apoptosis at 24h. Cell viability as well as apoptosis assays were carried out using a varied concentration of rfhSP-D (5, 10 or 20 µg/ml) and different timepoints (12, 24 and 48h), but only 20 µg/ml concentration of rfhSP-D was effective in inducting the optimal cell viability reduction and apoptosis induction at 24h. rfhSP-D treated BT474 and SKBR3 cells showed a significant reduction in viable cells compared to untreated control (cells only), as evident from trypan blue exclusion and MTT assay (data not shown). However, no effect of rfhSP-D was seen on triple negative (BT20) cell line in terms of cell viability. Recent studies have shown the ability of the CRD region of human SP-D to interact with N-glycans of EGFR, resulting in down-regulation of EGF signalling in A549 [23]. It is likely that rfhSP-D mediated apoptosis is due to HER2 expression on BT474 and SKBR3 cells; a lack of it seems to increase BT20 cells resistant to rfhSP-D effect. Whether HER2 is implicated in mediating rfhSP-D induced apoptosis is under investigation. It is worth mentioning here that this study was aimed at further evaluating the anti-tumour properties of rfhSP-D using breast cancer cell lines. Our previous study revealed that both full-length human SP-D molecule (516 kDa) as well as rfhSP-D (~60 kDa) induced comparable level of apoptosis using a range of pancreatic cancer cell lines [17].

The ability of rfhSP-D to induce apoptosis at 24h in SKBR3 and BT474 cell lines was evident from a significant increase in the number of Annexin V-/PI positive breast cancer cells examined via flow cytometry and fluorescence microscopy. The cell surface of the healthy cells is composed of asymmetrically distributed lipids on the inner and outer surface of the plasma membrane. Phosphatidylserine (PS) is one of these lipids, which is restricted to the inner surface of the plasma membrane, thus, is only exposed to the cytoplasm of the cell. However, when apoptosis is triggered by stress factors, lipid asymmetry is lost and PS translocate to the outer leaflet of the plasma membrane [45]. Annexin V is a 36-kDa calcium-binding intracellular protein that binds to PS [45]. Thus, Annexin V can also stain cells undergoing necrosis due to ruptured membrane that permits the access of Annexin V to the entire plasma membrane. Western blotting analysis of rfhSP-D treated BT474 and SKBR3
Breast cancer cells revealed cleavage of caspase 9 and 3 at 12h treatment. Caspases are well-known cysteine-aspartic proteases that play crucial role in apoptosis; Caspase 9 is triggered by cellular stress (e.g. DNA damage), via its binding to apoptotic protease-activating factor 1 (Apaf-1). Apaf-1/procaspase 9, in turn, triggers executioner caspases 3 and 7 [46]. Caspase 3 is an essential apoptotic executor, triggered by endoproteolytic cleavage at Asp175 which leads to inactivation of PARP (a known marker of apoptosis) [45,47]. These findings indicated that rfhSP-D mediated apoptosis induction in SKBR3 and BT474 cell lines is likely to occur via the intrinsic apoptotic pathway [48]. Recently, an integral role of rfhSP-D in innate immune surveillance against prostate cancer has been reported. rfhSP-D mediated apoptosis has been suggested to take place by two distinct mitochondrial apoptotic mechanisms [19]. It is also possible that p53 upregulation in rfhSP-D treated BT474 and SKBR3 cell lines may lead to downregulation of pAkt pathway, resulting in increased levels of Bad, Bax, triggering the release of cytochrome c, caspase 9 cleavage and apoptosis induction. Furthermore, previous studies have also reported the interaction of SP-D with HMGA1, CD14, CD91-calreticulin complex, SIRPα, and EGFR [19,22,23,49]. The involvement of SP-D with these key molecules is likely to be relevant as a part of possible mechanism/receptor through which rfhSP-D is likely mediating its apoptotic functions on breast cancer cells. As reported in previous studies, CRD domain of SP-D may interact with pattern recognition receptors TLR-2, TLR-4 [50] and CD14 [22], which may block pro-inflammatory and pro-survival downstream signalling on breast cancer cells. However, further investigation is crucial to understand the involvement of CRD, and its possible interacting partners involved in triggering downstream mechanism of apoptosis induction.

During the development of breast tumorigenesis, the tumour cells interact with surrounding stroma to create a tumour microenvironment, supporting the growth, survival, and invasion of cancer cells. The ECM is an essential constituent of the niche, leading to progression of malignant tumour formation and proliferation. ECM is composed of a wide range of proteoglycans and glycosaminoglycans, which offers a structural support and promotes tissue organization, thus, contributing to cell survival, proliferation, invasion, angiogenesis, and immune cell infiltration [35,51]. HA is one of the specific components of ECM, which is synthesized as a large linear anionic polymer at the cell surface. The role of HA during inflammation-mediated breast cancer has been established [52,53]. In vitro studies have reported the ability of HA to regulate tumour cell migration and invasion, and tumour growth and progression in in vivo models [39]. Synthesis and accumulation of HA has been detected in invasive breast cancer cells compared to normal breast epithelial tissues [39]. Additionally, HA synthase 2 facilitates invasion in breast cancer cells [51]. Furthermore, tumour cells over-expressing HA synthase 2 has shown to enhance angiogenesis and stromal cell recruitment, suggesting that increased levels of HA in the tumour stroma support breast carcinoma [51]. Pro-tumorigenic roles of increased HA expression in breast tumours, and possible mechanisms through which HA might trigger tumour proliferation in an area of great interest. Therefore, we examined the interaction between rfhSP-D and HA, and its implication on the adhesion and proliferation of BT20, BT474 and SKBR3 breast cancer cells.

Direct binding ELISA revealed that rfhSP-D bound to HA in a dose-dependent manner. The ability of breast cancer cells to interact with HA-bound rfhSP-D was examined via a cell adhesion assay. Cell adhesion assay was performed to assess the effect of rfhSP-D or HA treatment on the ability of breast cancer cells to adhere. A greater cell adhesion binding pattern was observed with rfhSP-D alone, when compared to HA-bound-rfhSP-D or HA alone. This may be due to the interaction of rfhSP-D treated cells with apoptotic-related
receptors and pathways leading to apoptosis induction. Apoptotic and proliferative assays was also performed to investigate the effect of HA-bound-rfhSP-D in comparison to rfhSP-D alone. SKBR3 cells seeded on HA-bound-rfhSP-D resulted in an increased cell proliferation (1.7-fold), when compared to rfhSP-D alone. In the case of rfhSP-D treated BT474 cells, addition of HA resulted in 2-fold increase of proliferative cells. However, the percentage of proliferating cells in presence of HA-bound-rfhSP-D was not significantly different from HA alone or untreated SKBR3 and BT474 cells. Therefore, these findings suggest that pro-apoptotic effects of rfhSP-D are modulated by HA addition. However, there was no significance fold differences of cell proliferation in the case of BT20 cells among all the conditions, suggesting that these cells continue to grow, and not affected by rfhSP-D treatment alone or in conjunction with HA. It is also possible that addition of HA may modulate the interaction of rfhSP-D treated cells with pro-apoptotic signalling pathways, thereby, restricting the possibility of apoptosis induction. Thus, addition of HA could facilitate cell proliferation by interacting with proliferative signalling cascades (e.g. Ras-ERK and PI3K-Akt). Even though breast cancer tissues express SP-D (Figure 4A&B), presence of HA within the breast tumour microenvironment seems to provide a self-protective coat, thus, negating the anti-tumorigenic properties of SP-D. Since BT474 and SKBR3 cells, treated with rfhSP-D undergo apoptosis, and addition of HA to rfhSP-D enhances cell proliferation, it is likely that breast cancer cells can use HA in their tumour microenvironment as an escape mechanism from apoptosis induction triggered by rfhSP-D.

To further understand the mechanism of apoptosis induction by rfhSP-D treatment, the likely signalling pathways involved in tumour adhesion, proliferation and cell death was analysed. rfhSP-D treatment resulted in increased levels of p53 phosphorylation (at Ser15), possibly mediated by oxidative stress caused by rfhSP-D [17,49]. p53 is an essential transcriptional modulator of apoptosis and plays a crucial role in cellular response to DNA damage as well as other genomic alterations. Phosphorylated p53 and increased levels of P21/P27 expression may cause inactivation of cyclin B-cdc2 complexes that are crucial in regulating G2/M cell cycle transition, causing DNA repair or induction of apoptosis [17]. However, decreased levels of caspase 3 and p53 levels were detected in HA+ rfhSP-D treated cells, possibly suggesting its resistance to apoptosis by addition of HA. Although p21 trigger is p53-dependent in certain events such as DNA damage, but there are several scenarios in which p21 expression is independent of p53 status, such as normal tissue development and differentiation. p21 can lead to tumor evolution directing towards tumor growth by slowing down the accumulation of DNA damage. Induction of p21 expression has been shown to be crucial for promoting motility of tumor cells and tumorigenesis. Due to controversy roles of p21, it can be considered as an oncogenic protein and or tumor suppressor, depending on its cytoplasmic localization. The dual roles of p21 as an oncogenic or tumor suppressor makes it difficult to study the mechanisms involved in p53-p21 mediated apoptosis induction or cancer progression.

Targeting HA synthesis, accumulation, degradation, or HA-receptor interaction represent a potential therapeutic approach for treatment of breast and other cancers. The use of 4-MU to inhibit HA synthesis has shown to reduce breast cancer proliferation and migration [54–56]. [57]. 4-MU treatment has also been shown to inhibit intracellular and cell surface HA [57], in addition to suppression of Akt phosphorylation Use of hyaluronidases to block HA in the tumour microenvironment has also been considered as a potential therapeutic approach for cancer. Bacteriophage hyaluronidase has been shown to effectively inhibit growth, migration, and invasion of breast cancer cells [58]. Hyaluronidases treatment has also shown promising outcome in pancreatic cancer and is
currently being tested in clinical trials [59–61]. Furthermore, recombinant human Hyaluronidase (rHUPH20) has been shown to improve drug delivery of antibody-based trastuzumab targeted therapy in HER2+ breast cancer [62].

Involvement of rfhSP-D and its associated pro-apoptotic properties in vitro cancer models might be a novel therapeutic agent to target multiple cellular signalling pathways, including tumor cell survival factors, transcriptional factors, protein kinases and pro-apoptotic gene signatures. The mechanism which rfhSP-D induces apoptosis and inhibits tumor cell proliferation is tumor specific due to the differential effects on the cell types, and probably expression of putative receptors. The nature of interaction between HA+ rfhSP-D, and cellular receptors is currently under investigation. The function of SP-D in the biology of cancer is complex and is strongly dependent on the tumor microenvironment. As SP-D and HA are overexpressed in breast cancer and other carcinomas, targeting both HA and SP-D is clinically relevant to inhibit HA-mediated intracellular signalling that negates pro-apoptotic role of rfhSP-D. It is important to note that FL-SP-D purified from BT474 was not able to induce apoptosis in any of the three breast cancer cell lines. The plausible reasons could be that the tumor cells produce a dysfunctional protein owing to mutations, protein misfolding, and/or altered oligomerization.

In conclusion, rfhSP-D treatment was able to induce apoptosis in BT474, and SKBR3 cell lines involving intrinsic apoptotic pathway, while having no effect on triple negative BT20 cell line. In addition, HA-bound-rfhSP-D was able to restore cell growth, suggesting that these breast cancer cells may use HA as an escape mechanism to overcome pro-apoptotic induction of rfhSP-D. The mechanisms by which HA-bound-rfhSP-D restores cell proliferation are yet to be elucidated.

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Figure Legends

**Figure 1:** SDS-PAGE (A) and immunoblot profile (B) of purified recombinant form of Human Surfactant Protein D (rfhSP-D). Expression of rfhSP-D under bacteriophage T7 promoter, expressed as ~20kDa insoluble protein. Eluted fractions were passed through a maltose agarose column and appeared as a single band at ~20kDa. Immunoreactivity of affinity purified rfhSP-D was examined via wester blotting; lane 1: BSA as a negative control and lane 2: purified rfhSP-D (5 μg/ml). Binding of rfhSP-D (10 μg/ml) to breast cancer cell lines using fluorescence microscopy, following 1h incubation at 4°C (C&D). The nucleus of the cells was stained with Hoechst (1:10,000), and both untreated- cells only (C) and treated with rfhSP-D (D) cells were probed with polyclonal anti-human SP-D/FITC antibody (1:200). Membrane localisation of the bound proteins was only detected in the treated cells, while no FITC was detected in the un-treated control (cells only).

**Figure 2:** Flow cytometric analysis of apoptosis induction in breast cancer cells treated with 20 μg/ml of immobilised (A) and solution phase (B) rfhSP-D for 24h. 12,000 cells were acquired and plotted for both annexin V/FITC and DNA/PI staining. The significance difference was seen among treated and untreated (cells only) samples, as evident by the shift in the fluorescence intensity. The data were expressed as mean of three independent experiments (n=3) done in triplicates ± SEM. Statistical significance was established using the unpaired one-way ANOVA test (**p < 0.01 and ****p < 0.0001). The percentage of apoptosis was calculated by normalizing the treated cells with their untreated counterparts. Fluorescence microscopy analysis of apoptosis induction in breast cancer cells treated with rfhSP-D (20 μg/ml) for 24h, using an annexin V with propidium iodide (PI) staining kit; untreated cells only controls (C), and rfhSP-D treated cells (D). The nucleus was stained with Hoechst (1:10,000), and the cell membrane was stained positively with annexin V and PI (1:200) in treated cell lines, suggesting that cells treated with rfhSP-D induced apoptosis at 24h, where translocation of PS into the outer plasma membrane was able to bind Annexin V due to loss of membrane integrity, and PI stain was taken in which stained the DNA of apoptotic cells. No Annexin V/PI staining was detected in untreated cells.

**Figure 3:** Caspase activation in breast cancer cell lines following rfhSP-D treatment at 24h. Western blot analysis of cleaved caspase 3 was observed at ~17 kDa following rfhSP-D treatment at 24 h (A).Western blot analysis showing activation of cleaved 9 was also shown by western at ~37 kDa (B), suggesting the trigger of intrinsic pathway.

**Figure 4:** SP-D and Hyaluronic acid (HA) presence in different histotypes of neoplastic breast (A-L), and normal ductal mammary epithelium (M-O) (A, D, G and J) (A). A high variability of SP-D expression between different histotypes is showed, although the positivity of the signal is always attributable to neoplastic cells, as can be observed from the higher resolution images in the right part of the figure (B, E, H and K). AEC (red) chromogen was used to visualize the binding of anti-human SP-D antibodies. (C, F, I and L) Histochemical staining with Alcian Blue highlighted HA distribution in breast cancer tissue sections; in particular, the staining was visible in tumour-associated stroma. Immunohistochemical and histochemical analysis in Luminal-B and Her2+/ER-/PR-breast carcinoma sections of SP-D (A-F) and HA (G-L) expression respectively (B). A high variability of SP-D and HA expression within the same isotypes is showed. It is possible
to notice a slight inverse correlation between SP-D and HA expression. AEC (red) chromogen was used to visualize the binding of anti-human SP-D antibodies, whereas histochemical staining with Alcian Blue for HA distribution.

Figure 5: Interaction of rfhSP-D with HA. Binding of varied concentration of rfhSP-D to Hyaluronic Acid (20 µg/ml) by ELISA (A). The effects of rfhSP-D on Breast cancer cell lines adhesion (B). Breast cancer cells were labelled with FAST Dil fluorescent dye and allowed to adhere to 96 microtiter wells pre-coated with HA, SP-D and BSA. The data is expressed as mean of three independent experiments. The adhesion of cells was measured after 35 minutes of incubation at 37°C under 5% CO₂. Results are expressed as adhesion percentage with reference to a standard curve established using an increasing number of FAST Dil labelled cells. The data were expressed as mean of three independent experiments (n=3) done in triplicates ± SEM. Statistical significance was established using the unpaired one-way ANOVA test (**p < 0.01 and ***p < 0.001). The statistical analysis was performed between rfhSP-D and HA+ rfhSP-D treated breast cancer cells.

Figure 6: Apoptosis induction of BT20, BT474 and SKBR3 cell lines following HA challenge in the presence and absence of rfhSP-D (A). The data were expressed as mean of three independent experiments (n=3). The significance difference was seen among treated and untreated samples, as evident by the shift in the fluorescence intensity. Staurosporine (1 µM/ml) was used as a positive control. Proliferative effects of rfhSP-D treatment on BT474 and SKBR3 breast cancer cell lines (B). BT474 and SKBR3 cells were seeded in wells pre-coated with HA, HA+ rfhSP-D, and rfhSP-D alone. The percentage of proliferative cells was evaluated by staining with mouse anti-human Ki-67 antibody and Ki-67 stained cells were measured via flow cytometry. The data were generated from at least three independent experiments (n=3) and presented as mean ± SD (*p<0.1, **p < 0.01 and ****p < 0.0001). The statistical analysis was performed between rfhSP-D and HA+ rfhSP-D treated breast cancer cells. The secretion levels of FL-SP-D was confirmed and analysed via western blotting (C).Culture medium collected from BT20, BT474, and SKBR3 cell lines were passed through a maltose Sepharose column, and the eluted fractions were validated via western blotting; FL-SP-D was detected at ~43kDa only for BT474. Both BT20 and SKBR3 cell lines did not secrete any FL-SP-D. Secreted FL-SP-D by BT474 was tested for its ability to induce apoptosis in these cells lines (D). No effect of secreted FL-SP-D was seen in terms of the cell viability and apoptosis induction.

Figure 7: Intracellular signalling to show phosphorylation of p53 in rfhSP-D treated BT20 and SKBR3 cell lines (A). Breast cancer cell lines were allowed to adhere to HA or HA-bound-rfhSP-D and the phosphorylation status of p53 was evaluated using total cell lysates with PathScan Antibody Array Kit (Cell Signalling). Data were generated from at least three independent experiments and presented as mean ± SD. rfhSP-D treatments results in upregulation of p21 and p27 cell cycle inhibitors (B). BT20, BT474 and SKBR3 (0.4 × 10⁶) cells were seeded in a 6 well plate pre-coated with rfhSP-D and HA+ rfhSP-D. The treated cells were lysed and pelleted down. The pelleted cells were subjected to RNA isolation, followed by cDNA synthesis and qPCR. Comparative quantification method was performed to calculate the efficiencies of each gene for each individual PCR reaction and is based on the second differential maximum method or take-off analysis. The take-off results obtained with p21/p27 primers were normalized with the
house-keeping gene TBP results. qPCR assay was conducted in triplicates, and error bars represents ± SEM. Unpaired-one-way ANOVA test was used to determine the significance; *p < 0.05, **p < 0.01 and ***p < 0.001,(n = 3). The statistical analysis was performed between rfhSP-D and HA+ rfhSP-D treated breast cancer cells.
Table 1: Flow cytometric analysis of apoptosis induction in breast cancer cells treated with 20 μg/ml of immobilised and solution phase rfhSP-D for 24h

|                | Immobilised Phase | Solution Phase |
|----------------|-------------------|----------------|
|                | Background Apoptosis (%) | Normalized Apoptosis with rfhSP-D (%) | Normalized Apoptosis with Staurosporine (%) | Background Apoptosis (%) | Normalized Apoptosis with rfhSP-D (%) | Normalized Apoptosis with Staurosporine (%) |
| BT20           | 5                 | 7              | 70              | 5                 | 12              | 70              |
| BT474          | 8                 | 61             | 70              | 8                 | 34              | 70              |
| SKBR3          | 4                 | 68             | 70              | 4                 | 53              | 70              |
Figure 1A&B
Figure 1C
Figure 1D
Figure 2A&B
Figure 2C
Figure 2D

Treated with rfhSP-D

**BT20**

**BT474**

**SKBR3**

PI-CY3  |  Annexin V-FITC  |  Hoechst  |  Merged

10 μm
Figure 3

A

Cleaved Caspase 3
~17 kDa

SKBR3  BT474  BT20

B

Cleaved Caspase 9
~37 kDa

SKBR3  BT474  BT20
Figure 4A
Figure 5A&B
Figure 6A&B
Figure 6C&D

C

D
Figure 7A&B
