

1 **Pathological significance and prognostic value of Surfactant Protein D in cancer**

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46 **ABSTRACT**

47 Surfactant protein D (SP-D) is a pattern recognition molecule belonging to the Collectin
48 (collagen-containing C-type lectin) family that has pulmonary as well as extra-pulmonary
49 existence. In the lungs, it is a well-established opsonin that can agglutinate a range of microbes,
50 and enhance their clearance via phagocytosis and super-oxidative burst. It can interfere with
51 allergen-IgE interaction and suppress basophil and mast cell activation. However, it is now
52 becoming evident that SP-D is likely to be an innate immune surveillance molecule against
53 tumor development. SP-D has been shown to induce apoptosis in sensitized eosinophils derived
54 from allergic patients and a leukemic cell line via p53 pathway. Recently, SP-D has been shown
55 to suppress lung cancer progression via interference with the epidermal growth factor signaling.
56 In addition, a truncated form of recombinant human SP-D has been reported to induce apoptosis
57 in pancreatic adenocarcinoma via Fas-mediated pathway in a p53-independent manner. To
58 further establish a correlation between SP-D presence/levels and normal and cancer tissues, we
59 performed a bioinformatics analysis, using Oncomine dataset and the survival analysis platforms
60 Kaplan-Meier plotter, to assess if SP-D can serve as a potential prognostic marker for human
61 lung cancer, in addition to human gastric, breast and ovarian cancers. We also analyzed
62 immunohistochemically the presence of SP-D in normal and tumor human tissues. We conclude
63 that (1) in the lung, gastric and breast cancers, there is a lower expression of SP-D than normal
64 tissues; (2) in ovarian cancer, there is a higher expression of SP-D than normal tissue; and (3) in
65 lung cancer, the presence of SP-D could be associated with a favorable prognosis. On the
66 contrary, at non-pulmonary sites such as gastric, breast and ovarian cancers, the presence of SP-
67 D could be associated with unfavorable prognosis. Correlation between the levels of SP-D and
68 overall survival requires further investigation. Our analysis involves a large number of dataset;
69 therefore, any trend observed is reliable. Despite apparent complexity within the results, it is
70 evident that cancer tissues that produce less levels of SP-D compared to their normal tissue
71 counterpart, are probably less susceptible to SP-D-mediated immune surveillance mechanisms
72 via infiltrating immune cells.

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77 **INTRODUCTION**

78 Surfactant protein D (SP-D) is a collagenous glycoprotein encoded by *SFTPD* gene belonging to
79 the collectins family (1). Like other members of the collectin family, SP-D has a primary subunit
80 structure that comprises of an N-terminal cysteine-rich region, a triple-helical collagen-like
81 domain, an α -helical coiled neck domain, and a C-terminal C-type lectin domain (also called
82 carbohydrate recognition domain (CRD) (2). Each subunit of human SP-D comprises three
83 identical polypeptide chains of 43 kDa, which is assembled into a tetrameric structure with 4 of
84 the homotrimeric subunits linked via their N-terminal regions, but trimers, dimers and monomers
85 also exist. Tetrameric structures can undergo further oligomerization to give SP-D multimers that
86 could contain up to 96 individual chains. SP-D was originally described in association with
87 pulmonary surfactant; in the lung, it is synthesized and secreted by type II alveolar cells and non-
88 ciliated bronchiolar epithelial cells. It has a key role in the maintenance of surfactant homeostasis
89 by reducing surface tension (3). Reduced SP-D expression or genetic variations (single
90 nucleotide polymorphism) have been associated with an increased risk of respiratory diseases (4,
91 5).

92 Extra-pulmonary existence of SP-D has also been reported. SP-D is also expressed by
93 epithelial cells lining various exocrine ducts, the mucosa of the gastrointestinal and genitourinary
94 tracts, the nasal cavity and in the brain (2). Furthermore, its presence has been demonstrated in
95 healthy lacrimal gland, conjunctiva, cornea, and nasolacrimal duct samples (6). Other studies
96 have shown the presence of SP-D in synovial fluid derived from patients with rheumatoid
97 arthritis (7).

98 In addition to its role in surfactant homeostasis, SP-D has a critical function as a regulator
99 of inflammation (3). In addition to its involvement in the recognition and neutralization of
100 pathogens which promotes aggregation/agglutination and inhibition of microbial growth (8), SP-
101 D is implicated in the clearance of necrotic and apoptotic cells (9). Thus, its function in the
102 recognition of non-self and altered self makes it a potent and versatile humoral pattern
103 recognition receptor (PRR) (10, 11). Furthermore, this molecule is able to inhibit the IL-12p40
104 production by macrophages (12). SP-D has also been described as a potent link between innate
105 and adaptive immune mechanisms also participates in the adaptive immune response (13-15).
106 Studies involving *in vivo* and *ex vivo* models of allergic inflammation revealed that SP-D can
107 alleviate pulmonary hypersensitivity via suppression of IgE levels, promotion of Th2 to Th1

108 polarization (16), apoptosis induction in sensitized eosinophils via p53-mediated pathway (17),
109 and inhibition of IgE synthesis by B cells (18). These studies highlighted a potential role of SP-D
110 as an immune surveillance molecule. It has recently been shown that SP-D also plays a role in
111 the control of lung cancer progression via epidermal growth factor signaling (19). Very recently,
112 Kaur *et al* have shown that a recombinant fragment of human SP-D, composed of homotrimeric
113 neck and C-type lectin domains, can induce apoptosis in pancreatic adenocarcinoma cell lines,
114 such as Panc-1 (p53^{mt}), MiaPaCa-2 (p53^{mt}), and Capan-2 (p53^{wt}), via Fas-mediated pathway
115 (20).

116 In the current study, we performed a bioinformatics analysis in order to investigate
117 whether SP-D can serve as a potential prognostic marker for human lung cancer. We extended
118 our investigation to several non-pulmonary sites such as human gastric, breast and ovarian
119 cancer. We used the Oncomine dataset and the survival analysis platforms Kaplan-Meier plotter.
120 Our results appear to suggest a likely pro-tumorigenic role of SP-D in gastric, breast and ovarian
121 cancers and an anti-tumor effect in lung cancer. Furthermore, we analyzed the presence of SP-D
122 in normal and tumor human tissues via immunohistochemistry. Differential expression of SP-D
123 was also investigated in human cells isolated from normal and tumour ovary tissues by Real-
124 Time PCR. This *in silico* study, if validated via a retrospective study at the protein level, could
125 be a step forward in ascertaining the importance of SP-D as a prognostic biomarker for different
126 cancers.

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128 **MATERIALS AND METHODS**

129 **Oncomine database analysis**

130 The expression level of *SFTPD* gene in various types of cancer was analyzed using Oncomine
131 (www.oncomine.org), a cancer microarray database and web-based data mining platform for a
132 new discovery from genome-wide expression analyses (21, 22). We compared the differences in
133 mRNA level between normal tissue and cancer. The mRNA expression level in neoplastic tissues
134 compared to the healthy tissues was obtained as the parameters of p -value < 0.001 , fold change $>$
135 all, and gene ranking in the top 10%. Information about the datasets used in this study is
136 summarized in Table I.

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138 **Kaplan-Meier plotter database analysis**

139 A Kaplan-Meier plotter database can assess the effect of 54,675 genes on survival using 10,461
140 cancer samples (5,143 breast, 1,816 ovarian, 2,437 lung and 1,065 gastric cancer patients with a
141 mean follow-up of 69/40/49/33 months) using probe sets on the HGU133 Plus 2.0 array. The
142 prognostic significance of SP-D expression and survival in breast, ovarian, lung and gastric
143 cancer was analyzed by Kaplan-Meier plotter (www.kmplot.com) (23). The hazard ratio with
144 95% confidence intervals and logrank p -value was also computed.

145

146 **Patients and Specimens**

147 8 fresh clinical specimens (4 normal ovarian epithelial tissues and 4 malignant ovarian epithelial
148 tumour tissues) were obtained from the Department of Gynaecology of IRCCS “Burlo
149 Garofolo”, in Trieste, Italy between 2016 and 2017. Oncological patients underwent laparoscopy
150 for diagnosis of pelvic mass whereas control patients underwent laparoscopy for other
151 indications. Tissue samples from patients were collected after informed consent following
152 approval of the ethical considerations by the Institutional Board of IRCCS “Burlo Garofolo”,
153 Trieste, Italy.

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155 **Immunohistochemical analysis**

156 For the immunohistochemical analysis, human normal and neoplastic tissues, including lung,
157 breast, ovary and stomach samples, were selected from the archives of the Department of
158 Pathology, University of Palermo. Immunohistochemistry (IHC) was performed using a polymer

159 detection method. Briefly, tissue samples were fixed in 10% v/v buffered formalin and then
160 paraffin embedded. 4 µm -thick tissue sections were deparaffinized and rehydrated. The antigen
161 unmasking technique was carried out using Novocastra Epitope Retrieval Solutions, pH 9 (Leica
162 Biosystems) in a PT Link pre-treatment module (Dako) at 98°C for 30 minutes. Sections were
163 then brought to room temperature (RT) and washed in PBS. After neutralization of the
164 endogenous peroxidase with 3% v/v H₂O₂ and Fc blocking by a specific protein block
165 (Novocastra, Leica Biosystems), samples were incubated overnight at 4°C with rabbit anti-
166 human SP-D (dilution 1:300) polyclonal antibodies (MRC Immunochemistry Unit, Oxford, UK).
167 Staining was revealed via polymer detection kit (Novocastra, Leica Biosystems) and AEC (3-
168 amino-9-ethylcarbazole, Dako, Denmark) substrate-chromogen. Slides were counterstained with
169 Harris Haematoxylin (Novocastra, Leica Biosystems). Sections were analysed under the Axio
170 Scope A1 optical microscope (Zeiss) and microphotographs were collected through the AxioCam
171 503 colour digital camera (Zeiss) using the Zen2 software.

172

173 **Cell isolation and culture**

174 Carcinoma cells (OvCa) and normal epithelial ovarian cells (OvEp) were isolated from biopsies
175 derived from ovarian tissue. The tissue was finely minced with a cutter, incubated with a
176 digestion solution composed by 0.5% trypsin (Sigma-Aldrich, Milan, Italy) and 50 µg/ml DNase
177 I (Roche, Milan, Italy) in Hanks' Balanced Salt solution with 0.5mM Ca²⁺Mg²⁺ (Sigma-Aldrich)
178 overnight at 4°C. Next, the enzymatic solution was changed with collagenase type 1 (1.5 mg/ml)
179 (Worthington Biochemical Corporation, DBA) diluted in Medium 199 with Hank's salts
180 (Euroclone Spa, Milan, Italy) for 30 min at 37°C. The digestion was blocked with 10% v/v fetal
181 bovine serum (FBS, GIBCO, Life Technology) and the cell suspension was filtered through a
182 100 µm pore filter (BD Biosciences, Italy). The cells were seeded in a 25 cm² flask, coated with
183 bovine gelatine, and cultured using Human Endothelial cells serum-free medium (HESF, Life
184 Technologies), 10% heat-inactivated FBS supplemented with EGF (10 ng/ml), basic FGF (20
185 ng/ml) and Penicillin-Streptomycin (Sigma-Aldrich). Fresh medium was replaced every 2-3
186 days. The cells were maintained at 37°C in humidified atmosphere with 5% v/v CO₂ and used at
187 their 5th to 8th passage for *in vitro* experiments.

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190 **RNA isolation, cDNA synthesis and Quantitative Real-Time PCR (qPCR)**

191 Total RNA was extracted from cells using EuroGOLD trifax (Euroclone), according to the
192 manufacturer's instructions, and reverse-transcribed as previously described (32). Quantitative
193 Real-Time PCR (qPCR) was carried out using a Rotor-Gene 6000 (Corbett, Qiagen, Ancona,
194 Italy) using iQ SYBR Green Supermix (Applied Biosystems, Milan, Italy). The sequences of the
195 primers used for amplification of TataBox Binding Protein (TBP) housekeeping gene are:
196 Forward 5'-GAGCCAAGAGTGAAGAACAGTC-3'; Reverse 5'-
197 GCTCCCCACCATATTCTGAATCT-3'. The sequences of SP-D primers are: Forward 5'-
198 AGGCTGCTTTCCTGAGCATGAC-3'; Reverse 5'-CCATTGGTGAAGATCTCCACACAG-
199 3'. The melting curve was recorded between 55°C and 99°C with a hold every 2s. The relative
200 amount of gene production in each sample was determined by the Comparative Quantification
201 (CQ) method supplied as part of the Rotor Gene 1.7 software (Corbett Research) (34). The
202 relative amount of each gene was normalized with TBP and expressed as arbitrary units (AU)
203 considering 1 AU obtained from fully differentiated macrophage used as calibrator.

204

205 **Statistical analysis**

206 Survival curves were generated by the Kaplan-Meier plots. All results are displayed with *p*
207 values from a logrank test. *P-values* < 0.05 were considered significant. Similarly, with
208 Oncomine, the statistical significance of data (*p-values*) was provided by the program.

209

210 **RESULTS**

211 **Clinical significance of SP-D expression in lung cancer**

212 We initially compared the differences in the mRNA level of SP-D between neoplastic and
213 healthy tissues using the Oncomine platform. While analyzing Bhattacharjee's, Hou's and
214 Garber's datasets, we detected a significantly lower SP-D mRNA expression in lung
215 adenocarcinoma, squamous cell carcinoma, large cell carcinoma, small cell carcinoma and tumor
216 carcinoid, compared to the normal lung tissue (Figure 1A, *p*<0.05; Supplemental Figure S1,
217 *p*<0.05). We subsequently performed a bioinformatic analysis of SP-D mRNA expression using
218 the Kaplan-Meier plotter dataset. As shown in Figure 1B, SP-D mRNA expression was
219 positively related to an overall survival rate of the patients with lung cancer, stratified into lung
220 adenocarcinoma and squamous cell carcinoma (*p*<0.05).

221 Immunohistochemical staining for SP-D confirmed a differential expression in healthy
222 and neoplastic pulmonary parenchyma. Moreover, in lung adenocarcinoma and squamous cell
223 lung carcinoma tissues, we observed a lower expression of SP-D within its microenvironment
224 compared to the healthy pulmonary parenchyma (Figure 2).

225

226 **Pathological significances of SP-D mRNA expression in gastric, breast and ovarian cancers**

227 The bioinformatics analysis on SP-D mRNA expression in gastric cancer via Cho's and
228 DErrico's datasets showed its higher expression in healthy gastric mucosa compared to its
229 malignant counterpart, stratified into intestinal, diffuse, and mixed-type adenocarcinoma by
230 Lauren's classification (Figure 3A, $p<0.05$; Supplemental Figure S2, $p<0.05$). According to the
231 data from Kaplan-Meier plotter, SP-D mRNA expression was negatively related to an overall
232 survival rate of the patients with gastric cancer (Figure 3B, $p<0.05$). If stratified by Lauren's
233 classification, SP-D mRNA expression had a statistically significant association with intestinal-
234 type adenocarcinoma, whereas no association with diffuse- and mixed-type adenocarcinomas
235 was found (Figure 3C, $p<0.05$). A higher expression of SP-D was negatively correlated with an
236 overall survival rate in the patients without distant metastasis, Her2-negative and only intestinal-
237 type adenocarcinoma (Figure 3D, $p<0.05$).

238 The information regarding the SP-D mRNA expression in breast cancer was obtained
239 from Zhao's, TCGA's and Curtis's datasets, which showed that *SFTPD* was expressed at a lower
240 level in invasive ductal breast carcinoma, male breast carcinoma and breast phyllodes tumor,
241 compared to normal breast tissues (Figure 4A, $p<0.05$; Supplemental Figure S3A, $p<0.05$).
242 According to the data from Kaplan-Meier plotter, *SFTPD* expression was negatively linked to the
243 high overall survival rate in breast cancer patients with Luminal-A grade-1 and grade-2 cancers
244 (Figure 4B, $p<0.05$; Supplemental Figure S3B, $p<0.05$). No correlation between SP-D mRNA
245 expression and overall survival rate was observed in patients with the other characteristics
246 (Luminal-B, HER2⁺, Basal, grade-3, mutated p53, wild-type p53).

247 Using immunohistochemistry, we observed a variable presence and distribution of SP-D
248 in normal tissues with respect to their cancer counterpart. In fact, immunohistochemical analysis
249 performed on either healthy or neoplastic gastric mucosa highlighted a significantly reduced
250 expression of SP-D in the intestinal-type adenocarcinoma compared to gastric control tissue
251 (Figure 5A and C). Likewise, a higher expression of SP-D in the normal mammary parenchyma

252 was detected compared to that observed within microenvironment within the invasive ductal
253 breast carcinoma, Luminal-A (Figure 5B and D).

254 We collected the results from Yoshihara's and TCGA's datasets and analyzed *SFTPD*
255 expression in ovarian cancer. We highlighted a lower expression of *SFTPD* mRNA expression in
256 normal ovary than in serous cystadenocarcinoma (Figure 6A, $p<0.05$). The Kaplan-Meier plotter
257 data, derived from stage -1 and -2 patients, showed a negative ratio between *SFTPD* expression
258 and either overall or progression-free survival rates of patients with serous cystadenocarcinoma
259 (Figure 6B, $p<0.05$). However, no correlation was observed between *SFTPD* expression and
260 these parameters (overall or progression-free survival rates) of patients with stage -3 and -4
261 ovarian cancer.

262

263 **Prevalence of SP-D expression in the microenvironment of ovarian cancer**

264 The mRNA expression of SP-D has been also evaluated by Real Time PCR in primary cells
265 isolated from 4 samples of human ovarian serous cystadenocarcinoma and 4 samples of normal
266 ovarian tissues. As shown in figure 7A, the cells isolated from ovarian serous
267 cystadenocarcinoma tissues expressed more SP-D compared to the normal tissue, confirming
268 the data obtained with the bioinformatics analysis. Immunohistochemical analysis also revealed
269 the presence and the distribution of SP-D in the normal ovary where it appeared to be localized
270 in the ovarian epithelium lining and in the serous cystadenocarcinoma. In addition, we detected
271 differential expression in the normal and its malignant histotypes. Moreover, in the ovarian
272 context, it showed an enrichment of SP-D expressing cells within the tumor microenvironment
273 compared to the control tissue (Figure 7B and C).

274

275 **DISCUSSION**

276 The importance of SP-D in the regulation of the inflammation and homeostasis and in the
277 protection against infection and allergens in the lung and at a range of extra-pulmonary sites is
278 well documented (24, 25). However, there are recent evidences to implicate SP-D as an immune
279 surveillance molecule against cancer (19, 20). In this study, we examined the potential
280 prognostic value of this protein in lung, gastric, breast and ovarian cancers. We focused our
281 attention on these tumor types because we performed a bioinformatics analysis using the Kaplan-
282 Meier plotter dataset, a manually curated database containing the information of 54,675 genes on

283 5,143 breast, 1,816 ovarian, 2,437 lung and 1,065 gastric cancer samples. This is the most
284 updated and reliable dataset available that offers the possibility of stratifying the analysis based
285 on different tumor settings. The bioinformatics analysis highlighted a favorable prognostic effect
286 of SP-D mRNA expression in the lung cancer, both in adenocarcinoma and squamous cell
287 carcinoma; on the contrary, an unfavorable prognostic effect in gastric, ovarian and breast cancer
288 was revealed. In particular, SP-D mRNA expression showed a negative correlation with the
289 intestinal-type gastric adenocarcinomas, grade-1 and grade-2 breast cancers and with stage -1
290 and -2 ovarian cancers. No significant correlation was showed with stage -3 and -4 in breast and
291 ovarian cancers.

292 Sin *et al* (26) have suggested earlier that low SP-D levels may be correlated to the
293 development of lung cancer. They observed a reduction of the concentration of SP-D in the
294 bronchoalveolar lavage fluid of heavy smokers that was linked to bronchial dysplasia. More
295 recently, Hasegawa *et al* (19) showed the presence of SP-D in lung cancer and demonstrated that
296 SP-D was able to interfere, via its CRD region, with the interaction between epidermal growth
297 factor (EGF) to EGF receptor (EGFR), a tyrosine kinase receptor of the ErbB family, causing
298 downregulation of the EGF induced signaling (19). This receptor is commonly altered in
299 epithelial tumors and its dysregulation leads to cell proliferation, angiogenesis, invasion, and
300 metastasis (27). Furthermore, it has been recently demonstrated that SP-D is also able to interact
301 with the mutant form of EGFR, inhibiting its ligand-independent dimerization (27). Very
302 recently, Kaur *et al* have reported the ability of a recombinant form of human SP-D to induce
303 apoptosis via TNF- α /Fas-mediated pathway in human pancreatic adenocarcinoma using Panc-1
304 (p53^{mt}), MiaPaCa-2 (p53^{mt}), and Capan-2 (p53^{wt}) cell lines. Treatment of these cell lines with a
305 recombinant form of truncated human SP-D (made up of neck and C-type lectin domains only)
306 for 24 h caused growth arrest in G1 cell cycle phase and triggered transcriptional upregulation of
307 pro-apoptotic factors such as TNF- α and NF- κ B. Translocation of NF- κ B from the cytoplasm
308 into the nucleus of pancreatic cancer cell lines was observed following treatment with SP-D. SP-
309 D treatment caused upregulation of pro-apoptotic marker Fas, which then triggered cleavage of
310 caspase 8 and 3. This study raises the possibility of using recombinant SP-D as a therapeutic
311 molecule against pancreatic cancer irrespective of their p53 phenotype (20).

312 The EGFR is commonly overexpressed in non-small cell lung cancer (in 89% squamous
313 cell carcinoma; 41% adenocarcinomas) (28), and therefore, it is considered a potential target for

314 cancer therapy (28); the presence of SP-D in these cancers could exert a protective role via
315 downregulation of the EGFR pathway. It has also been shown that serum level of SP-D reflects
316 its levels in the lung and that higher amount of SP-D in the serum correlated with better overall
317 survival in patients with EGFR mutant adenocarcinoma undergoing treatment with gefitinib, a
318 tyrosine kinase inhibitor (27).

319 Our study appears to highlight a more favorable prognosis for adenocarcinoma with
320 respect to squamous cell carcinoma. A possible explanation of this observation may be that
321 adenocarcinoma originates from peripheral airways progenitor cells that are able to produce SP-
322 D. Moreover, more SP-D production may be indicative of a more differentiated cancer.

323 *SFTPD*, together with a number of genes selectively expressed in the respiratory
324 epithelial cells, is under the control of the thyroid transcription factor 1 (TTF-1) (29, 30). A
325 recent meta-analysis showing that TTF-1 overexpression is related to a favorable prognosis for
326 NSCLC (non-small cell lung carcinoma) patients (31), appears to strengthen the results being
327 reported here.

328 Although the overexpression of the *EGFR* gene has also been reported in a variety of
329 other cancers including those of head and neck, ovary, cervix, bladder, esophagus, stomach,
330 brain, breast, endometrium and colon (32), the above-mentioned mechanisms cannot explain the
331 opposite results obtained via the bioinformatics analysis of Kaplan Mayer dataset for gastric,
332 ovarian and breast carcinomas, where SP-D showed an unfavorable prognostic effect. We think
333 that the unfavorable prognostic effect of SP-D in other tumor settings can be due to its direct or
334 indirect action on the immune population present in the tumor microenvironment (15). The
335 following mechanisms can explain the role of SP-D in determining a tumor microenvironment
336 favorable to tumor progression. For example, the protective effect of SP-D against breast cancer
337 cells can be negated by the presence of hyaluronic acid, which is abundantly present in the
338 microenvironment of a number of solid tumors (33) (Murugaiah, Bulla and Kishore, unpublished
339 data).

340 SP-D is able to reduce the expression of CD11c (15). CD11c is predominantly expressed
341 on dendritic cells, but also on effector cells in the local tumor microenvironment, such as some
342 macrophages, natural killer (NK) and activated T cells (34). It has been shown that low CD11c
343 expression indicates unfavorable prognosis in patients with gastric cancer (35).

344 SP-D can promote production of TNF- α and IFN- γ (16, 18, 36). The anti-tumor effects of
345 Th1 cells may reflect their known role in enhancing CD8⁺ T cell responses and activating
346 macrophages, through the secretion of TNF- α and IFN- γ . IFN- γ can increase tumor cell class I
347 MHC expression and sensitivity to lysis by NK cells and cytotoxic T lymphocytes (CTLs).
348 Besides, antigen-presenting cells such as macrophages and dendritic cells can directly activate
349 antigen-specific Th1 or CTLs, which can activate the anti-tumor immune response and are thus
350 associated with favorable prognosis of patients with many types of cancers (37, 38).

351 It has been demonstrated that SP-D binds to lymphocytes and suppress T cell
352 proliferation (14) via apoptosis induction in activated PBMCs. SP-D has been shown to enhance
353 expression of CTLA-4, a negative regulator of T cell activation and proliferation (39). In
354 addition, monocytes expressed CTLA-4, but only the lymphocytes treated with SP-D show a
355 significant overexpression of CTLA-4 (15). There is strong experimental and clinical evidence to
356 suggest that T cell responses to some tumors are inhibited by the involvement of CTLA-4, one of
357 the best-defined inhibitory pathways in T cells (40, 41). In fact, tumor-infiltrating T cells often
358 have a dysfunctional (exhausted) phenotype that is characterized by impaired effector functions
359 and increased expression of CTLA-4 and other inhibitory molecules (40, 41). Blockade of the
360 CTLA-4 pathways is now being widely used in the clinic to reverse the dysfunctional phenotype
361 of tumor-specific T cells and enhance their ability to kill tumor cells (41). In conclusion, SP-D,
362 increasing the expression of CTLA-4 also may contribute to the inhibition of the anti-tumor
363 immune responses.

364 SP-D is able to inhibit the IL-12p40 production by macrophages via the
365 SIRP α /ROCK/ERK signaling pathway (12). IL-12p40 is a component of IL-12p70 and IL-23,
366 and its regulation is important for both innate and adaptive immunity. IL-12p40 is a marker of
367 M1-like macrophages and data indicate that IL-12p40 may be contributing to inducing Th 1
368 polarization (42, 43). Macrophages derived from IL-12p40 deficient mice have a bias toward
369 M2-like polarization (42). The production of IL-12p40 by macrophages and dendritic cells is
370 associated with the ability to migrate to the lymph node and initiate T cell responses (44). We
371 think that SP-D repressing the expression of IL-12p40 in macrophages may maintain the steady
372 M2-like polarization and inhibit Th1 polarization.

373 SP-D is able to interact with the leukocyte-associated Ig-like receptor-1 (LAIR-1) (45),
374 also known as CD305. This molecule is a transmembrane glycoprotein and is expressed on

375 almost all immune cells and CD34⁺ hematopoietic progenitor cells. SP-D acts as a ligand for the
376 inhibitory receptor LAIR-1, which inhibits the function of multiple types of immune cells (45)
377 indicating that SP-D present in the tumour microenvironment may exert its immunomodulatory
378 effect and inhibit the anti-tumor immune responses through LAIR-1 activation. Thus, the context
379 of immune infiltration and composition of tumour microenvironment dictate the consequent
380 effects of SP-D, and hence, tumour progression or resistance.

381 In summary, our *in silico* analysis, if confirmed with a retrospective study at the protein
382 level, could highlight a possible role of SP-D as a novel marker for tumor prognosis in a range of
383 cancers. The presence of SP-D in cancer could be associated with a favorable prognosis in lung
384 cancer where it has been demonstrated to downregulate the EGF signaling and unfavorable
385 prognosis in non-pulmonary sites such as gastric, breast and ovarian cancers. We hypothesized
386 different possible mechanisms that this molecule could play in the tumor microenvironment on
387 the basis of the existing knowledge about the interaction of SP-D with the different immune
388 cells.

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406 ***Ethics Statement***

407 This study was carried out in accordance with the recommendations of governmental guidelines,
408 and approved by the CEUR (Comitato Etico Unico Regionale, FVG, Italy). with written
409 informed consent from all subjects. All subjects gave written informed consent in accordance
410 with the Declaration of Helsinki.

411

412 ***Author Contributions***

413 Conception and design: A. Mangogna and R. Bulla. Development of methodology: A. Gulino, C.
414 Agostinis and F. Romano. Acquisition of data: B. Belmonte and C. Agostinis. Analysis and
415 interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Tripodo,
416 A. Mangogna, B. Belmonte and C. Agostinis. Writing, review, and/or revision of the manuscript:
417 R. Bulla, U. Kishore, C. Agostinis and G. Ricci. Study supervision: R. Bulla.

418

419 ***Conflict of Interest Statement***

420 The authors declare that the research was conducted in the absence of any commercial or
421 financial relationships that could be construed as a potential conflict of interest.

422

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434

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Datasets	Study Description	Experiment Type
Bhattacharjee Lung	139 lung adenocarcinoma, 21 squamous cell lung carcinoma, 20 lung carcinoid tumor, 6 small cell lung carcinoma, and 17 normal lung samples were analyzed on Affymetrix U95A microarrays. Sample data includes type, age, M stage, max tumor percentage, N stage, primary/metastatic, recurrence, sex, site of metastasis, smoking rate (packs per year), stage, survival, and T stage.	mRNA
Hou Lung	91 non-small cell lung carcinoma and 65 adjacent normal lung samples were analyzed. Sample data includes age, sex, cancer sample site, and survival.	mRNA
Garber Lung	67 lung carcinoma samples of various types and 6 normal lung samples were analyzed on cDNA microarrays. Sample data includes type, grade, TNM stage, and survival.	mRNA
Cho Gastric	65 gastric adenocarcinoma, 19 paired surrounding normal tissue, and 6 gastrointestinal stromal tumor samples were analyzed. Sample data includes age, grade, stage, TNM stage, sex, and subgroup.	mRNA
DErrico Gastric	31 paired gastric carcinoma and adjacent normal gastric mucosa and 7 unmatched gastric carcinoma samples were analyzed. Sample data includes microsatellite status, age, sex, and TNM stage.	mRNA
Zhao Breast	Normal breast (n=3) and breast carcinoma (n=61) samples were analyzed on cDNA microarrays. Sample data includes tumor percentage, age, E-Cadherin status, estrogen receptor status, grade, HER2 status, lymph node metastasis status, and progesterone receptor status.	mRNA
TCGA Breast	532 invasive breast carcinoma, 61 paired normal breast tissue and 3 paired metastatic samples were analyzed. Sample data includes age, histology, TNM stage, ER/PR/ERBB2 status, sex, stage, and others. This dataset consists of Level 2 (processed) data from the TCGA data portal.	mRNA
Curtis Breast	1,992 breast carcinoma samples and 144 paired normal breast samples were analyzed for the METABRIC project. Sample data includes ER/PR/ERBB2 status, overall survival status and follow-up time, stage, grade, and others.	mRNA
Yoshihara Ovarian	43 ovarian serous adenocarcinomas and 10 normal peritoneum samples were analyzed. Sample data includes cancer sample site, stage, and sex.	mRNA
TCGA Ovarian	586 ovarian serous cystadenocarcinoma samples and 8 normal ovary samples were analyzed. Sample data includes age, stage, grade, survival, and others. This dataset consists of Level 2 (processed) data from the TCGA data portal.	mRNA

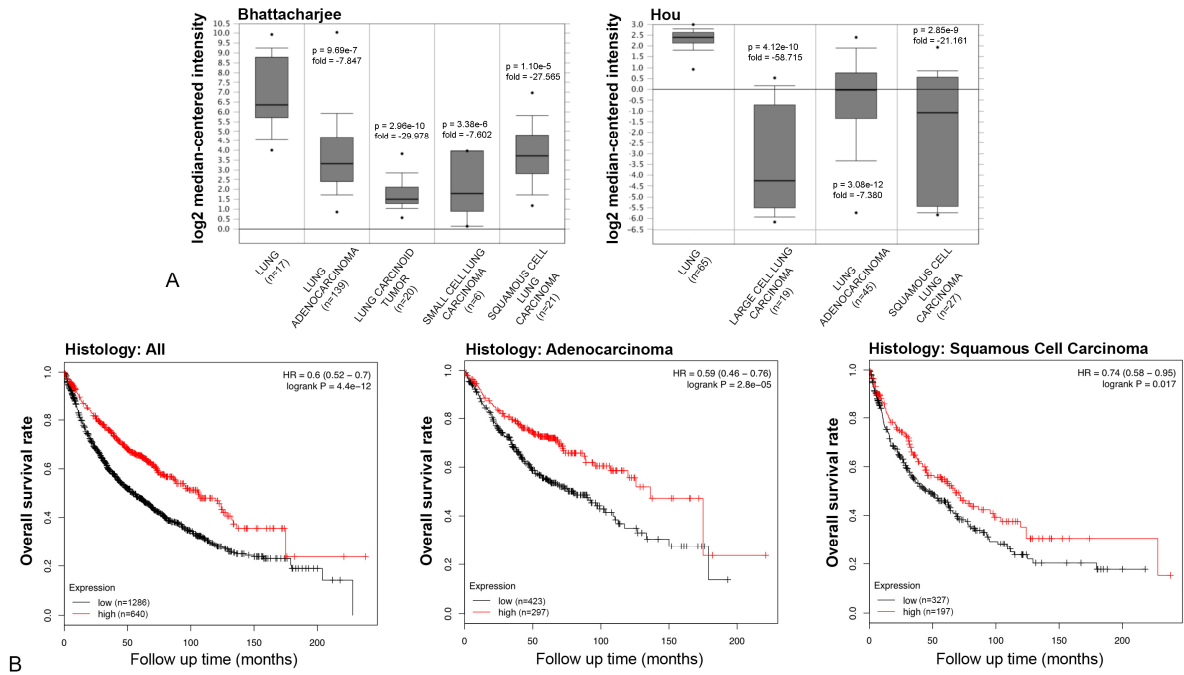
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592 **Table I. Data characteristics used in the bioinformatics analysis.**

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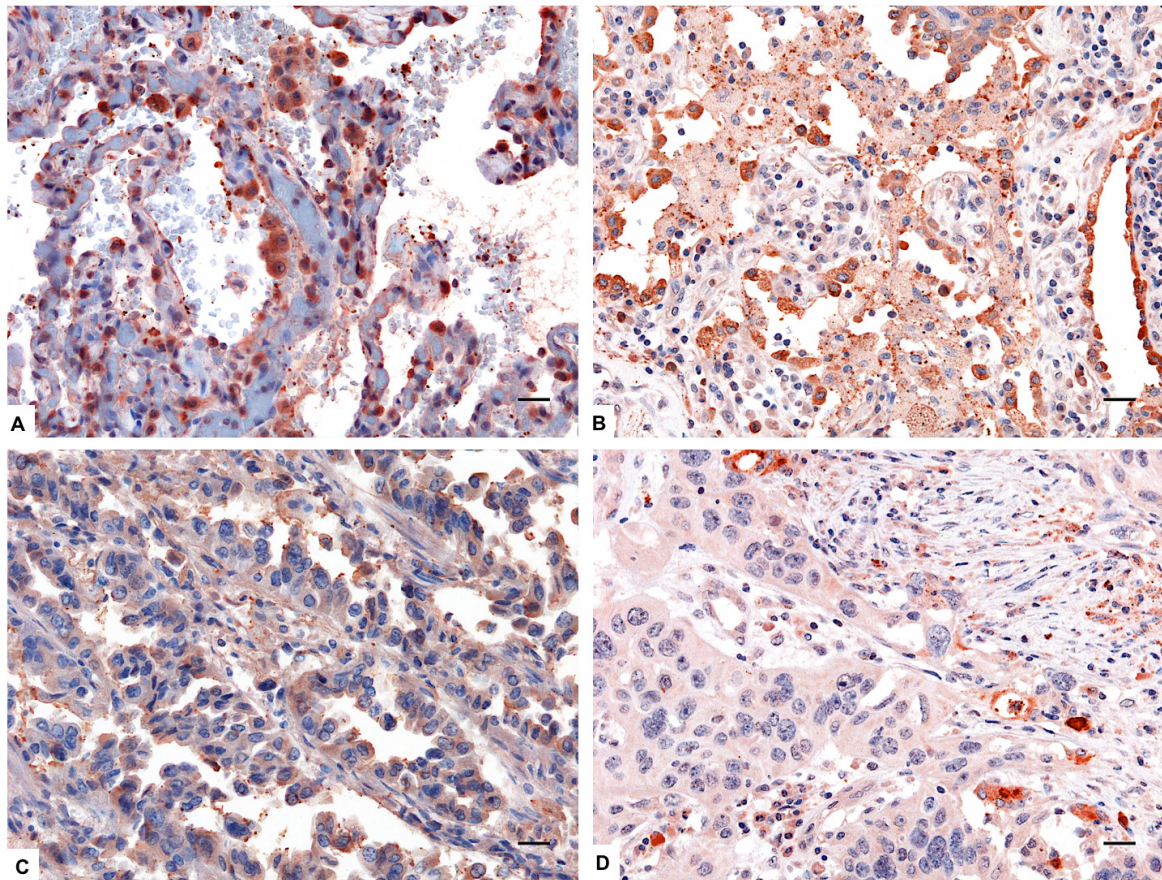
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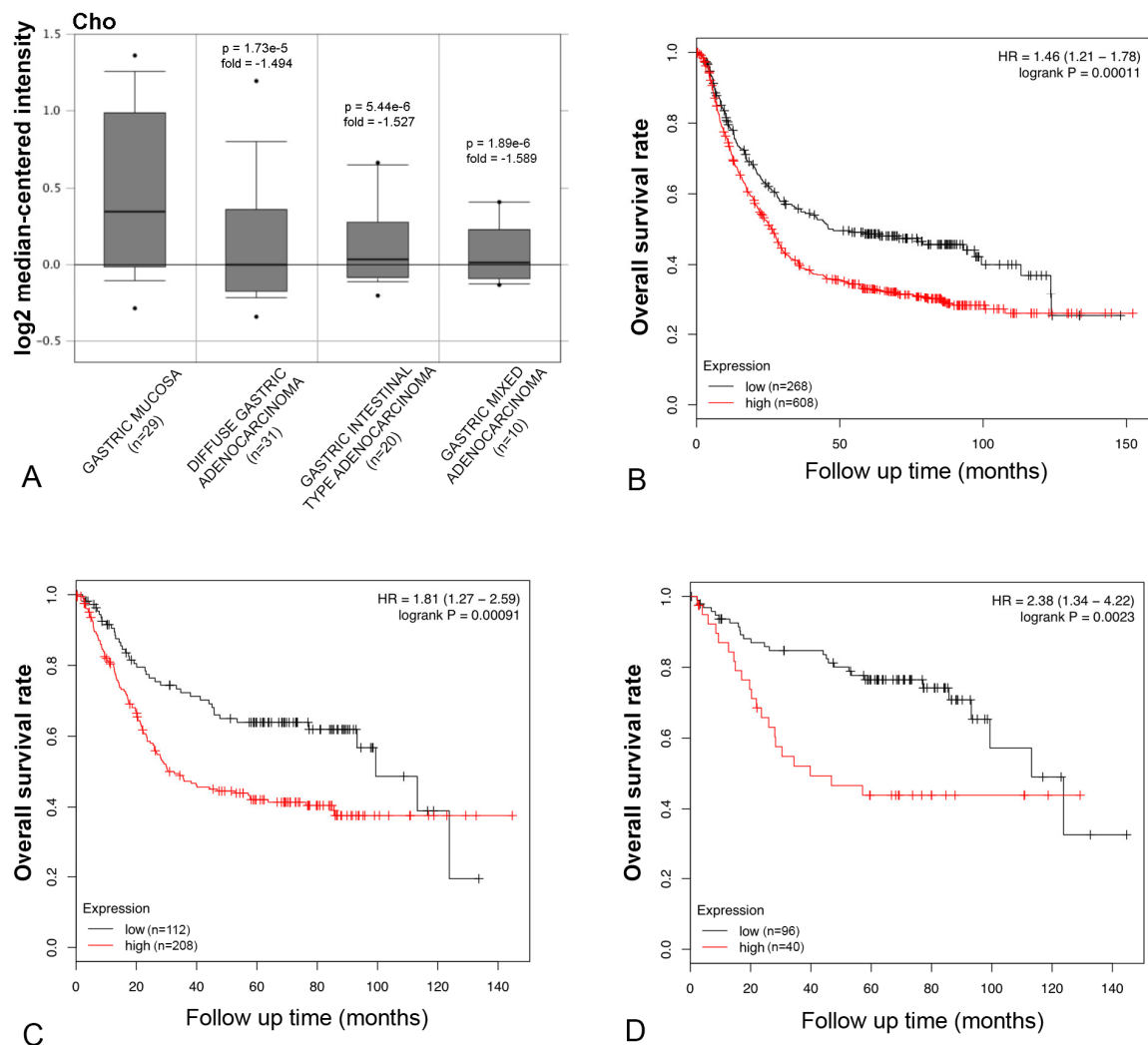
Figure 1. Pathological significance of SP-D expression in lung cancer. Bhattacharjee's and Hou's datasets were used for bioinformatics analysis to explore SP-D mRNA expression in the lung cancer. A lower SP-D mRNA expression was detectable in lung adenocarcinoma, squamous cell carcinoma, large cell carcinoma, small cell carcinoma and tumor carcinoid than in normal lung tissue (A, $p < 0.05$). According to the data from Kaplan-Meier plotter, SP-D mRNA expression was positively related to an overall survival rate of the patients with lung cancer, even stratified into lung adenocarcinoma and squamous cell carcinoma (B, $p < 0.05$). HR, hazard ratio.



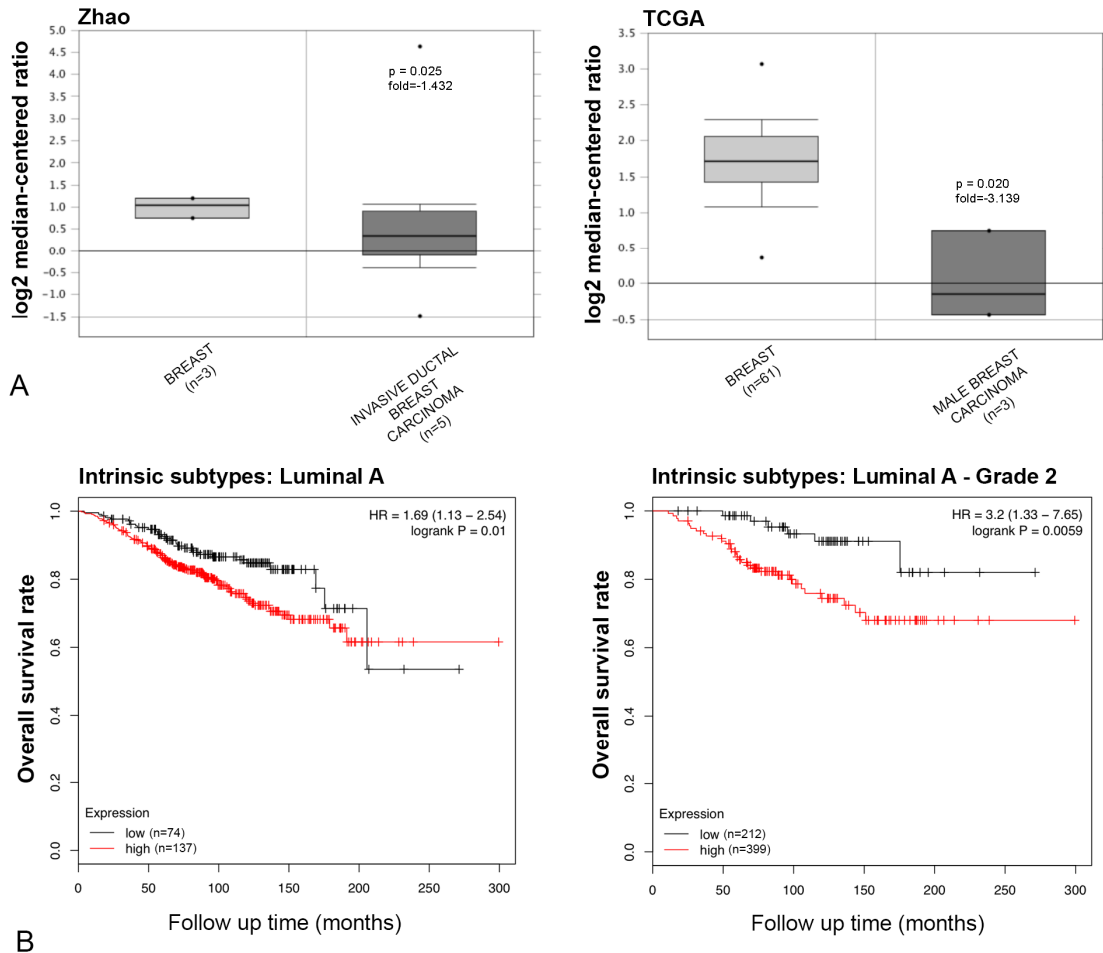
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625 **Figure 2. Immunohistochemistry analysis for SP-D in lung.** Compared to healthy lung (A –
626 **B**) a decreased expression of SP-D in squamous cell carcinoma (C) and adenocarcinoma of the
627 lung (D) was observed. Polymer detection system with AEC (red) chromogen was used; scale
628 bars, 50µm. Polymer detection system with AEC (red) chromogen was used; scale bars, 50µm.

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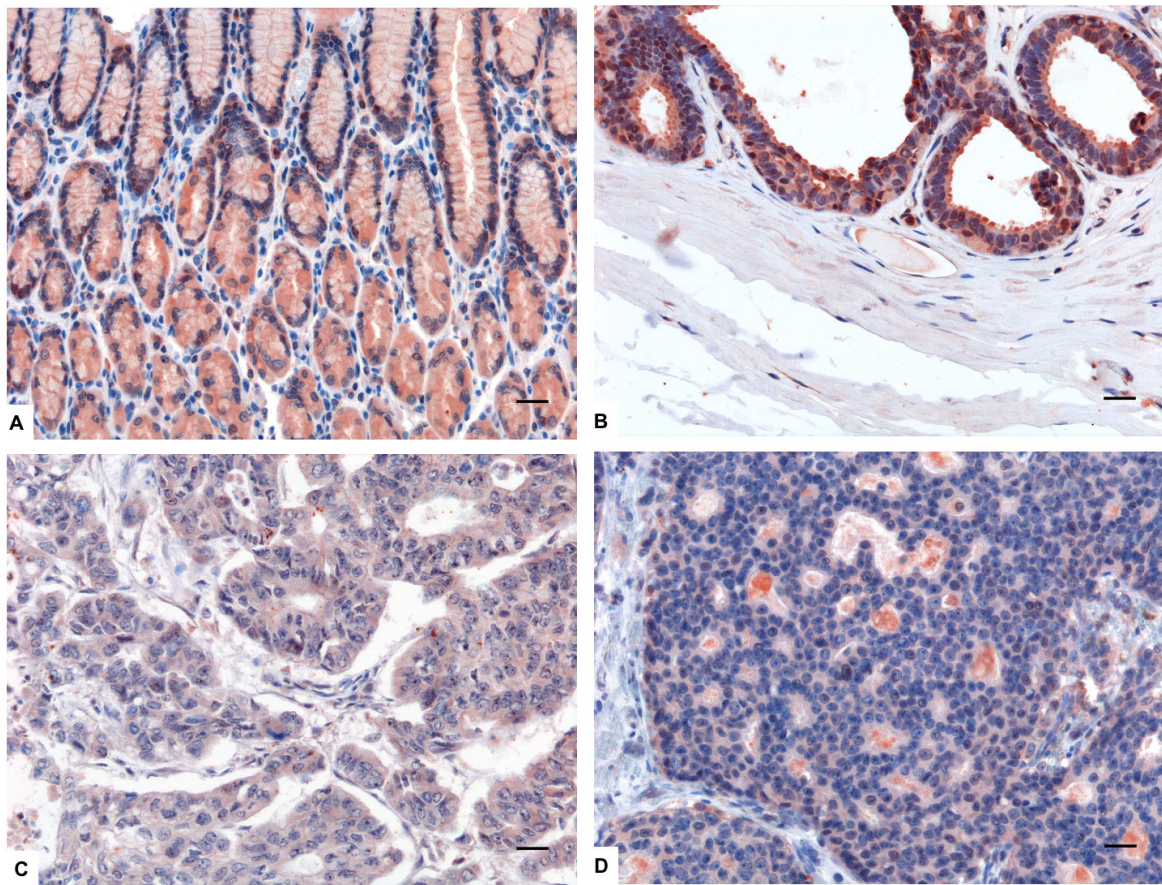


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 637 **Figure 3. Pathological significance of SP-D expression in gastric cancer.** Cho's dataset has
 638 explored SP-D mRNA expression in gastric cancer. A lower *SFTPD* expression was detectable
 639 in gastric cancer than that in normal mucosa, even stratified into diffuse-, intestinal-, and mixed-
 640 type adenocarcinomas by Lauren's classification (A, $p < 0.05$). According to the data from
 641 Kaplan-Meier plotter, SP-D mRNA expression was negatively related to an overall survival rate
 642 of the patients with gastric cancer (B, $p < 0.05$). If stratified by Lauren's classification, SP-D
 643 mRNA expression was negatively related to an overall survival rate in the patients with
 644 intestinal-type adenocarcinoma (C, $p < 0.05$), without distant metastasis and Her2-negative (D,
 645 $p < 0.05$). HR, hazard ratio.
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Figure 4. The clinicopathological significances of SP-D expression in breast cancer. Zhao's and TCGA's datasets have revealed that a lower SP-D mRNA expression in invasive ductal breast carcinoma and male breast carcinoma that in normal breast tissues (**A**, $p < 0.05$). There was a negative association between SP-D mRNA expression and a favorable prognosis in the breast cancer patients with Luminal-A only with grade -1 and -2 cancers, for Kaplan-Meier plotter (**B**, $p < 0.05$). HR, hazard ratio.



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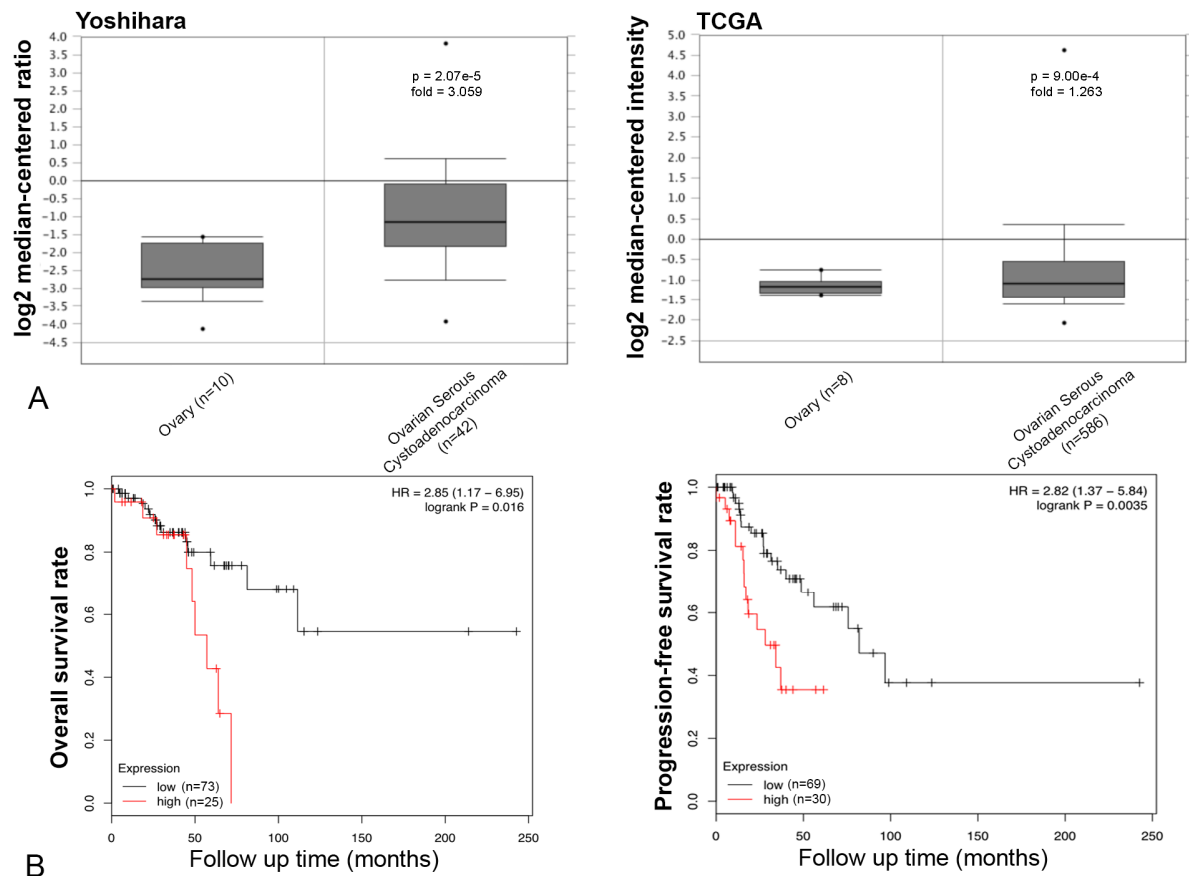
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Figure 5. Representative Immunohistochemical microphotographs of SP-D expression in the healthy gastric mucosa (A), and ductal mammary epithelium (B) and their malignant histotypes intestinal-type gastric adenocarcinoma (C) and invasive ductal breast carcinoma, Luminal-A (D). a decreased expression of SP-D in the intestinal-type gastric adenocarcinoma and invasive ductal breast carcinoma, Luminal-A respect to their normal counterparts can be observed. Polymer detection system with AEC (red) chromogen; scale bars, 50 μ m.

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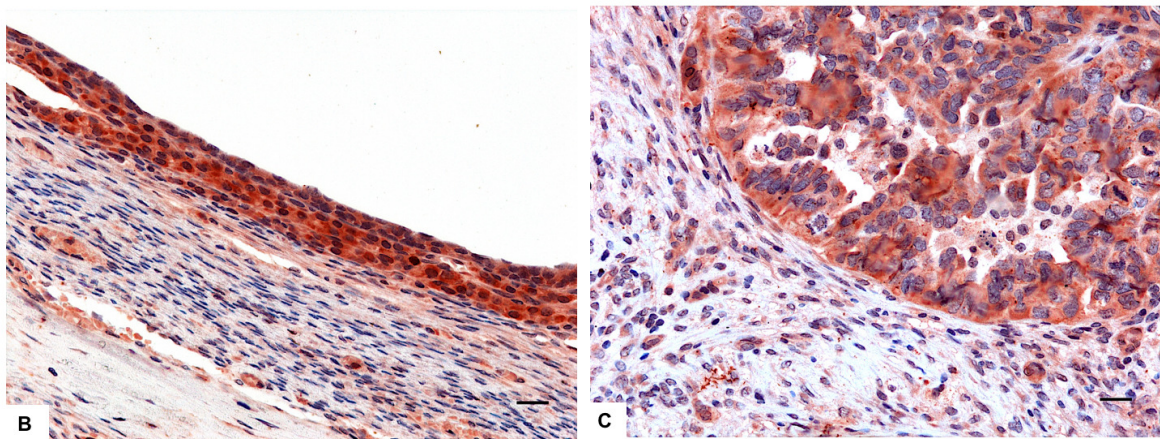
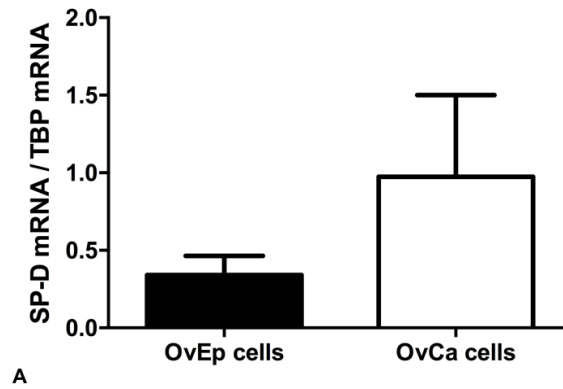


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683 **Figure 6. Pathological significance of SP-D expression in ovarian cancer.** Yoshihara's and
684 TCGA's datasets explored SP-D mRNA expression in ovarian cancer. A higher SP-D mRNA
685 expression was detectable in serous cystadenocarcinoma than that in normal ovary (A, $p < 0.05$).
686 According to the data from Kaplan-Meier plotter, SP-D mRNA expression showed a negative
687 relationship both overall or progression-free survival rates of patients with serous
688 cystadenocarcinoma, if stratified by stage-1 and -2 (B, $p < 0.05$). HR, hazard ratio.

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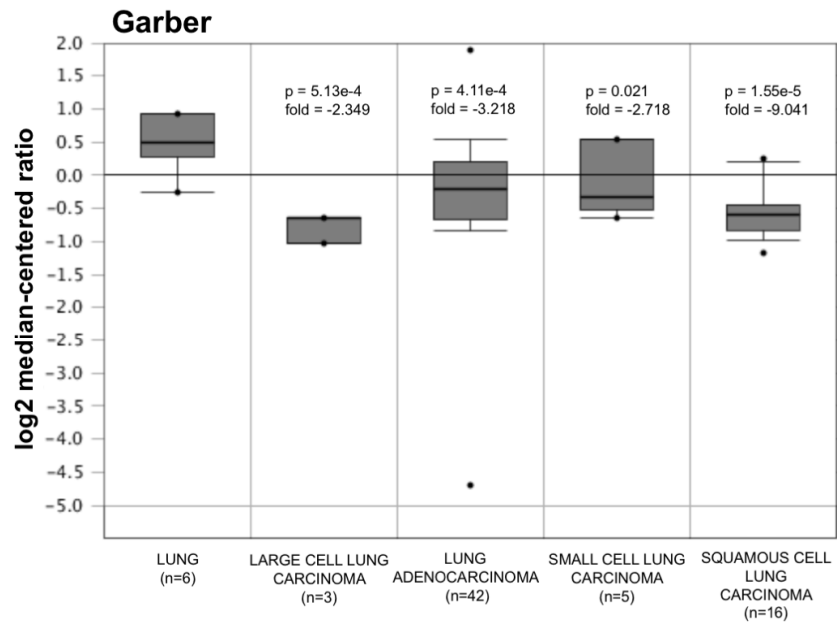
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Figure 7. **Figure 7.** Quantitative Real-Time PCR analysis of SP-D performed on the normal ovarian epithelium and the epithelial ovarian tumour (A). Representative microphotographs showing an enrichment in SP-D positive cells in the serous cystadenocarcinoma (B) compared to the normal ovarian epithelium (C). Polymer detection system with AEC (red) chromogen; scale bars, 50 μ m.

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715 **Supplemental Figure S1. SP-D expression in lung cancer.** Garber's dataset has explored SP-D
716 mRNA expression in the lung cancer. A lower SP-D mRNA expression was detectable in large
717 cell carcinoma, adenocarcinoma, squamous cell carcinoma and small cell carcinoma than in
718 normal lung tissue ($p < 0.05$).

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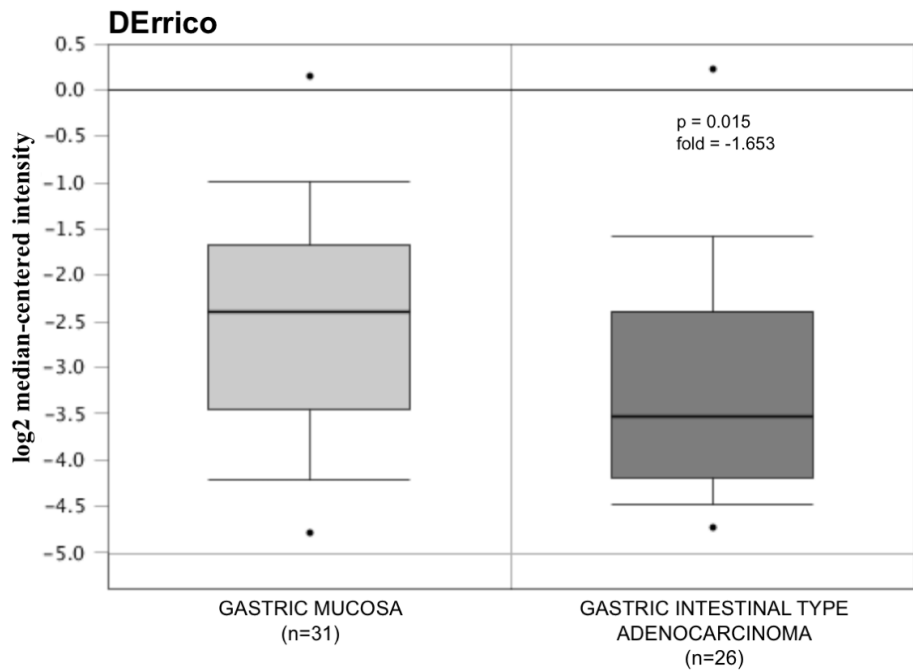
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730 **Supplemental Figure S2. SP-D expression in gastric cancer.** DErrico's dataset has revealed a
731 lower SP-D mRNA expression in intestinal-type adenocarcinoma than in normal mucosa
732 ($p < 0.05$).

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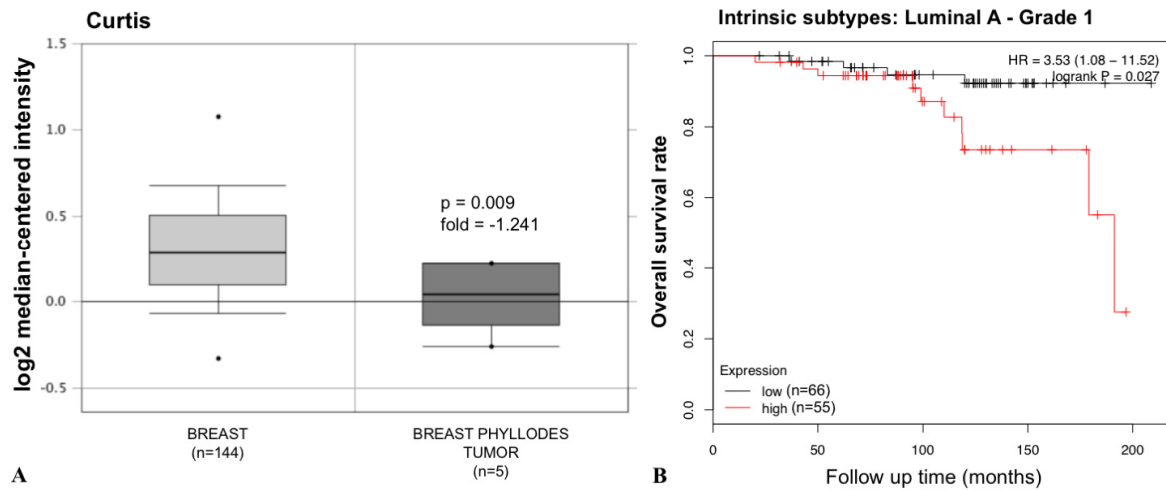
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744 **Supplemental Figure S3. Pathological significance of SP-D expression in breast cancer.**

745 Curtis's dataset has revealed a lower SP-D mRNA expression in phyllodes tumor than in normal
 746 breast tissue. (A, $p < 0.05$). There was a negative association between SP-D mRNA expression
 747 and a favorable prognosis in the breast cancer patients with Luminal-A with grade -1, for
 748 Kaplan-Meir plotter (B, $p < 0.05$). HR, hazard ratio.

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