1 # Expression of Spred2 in the urothelial tumorigenesis of the
2 urinary bladder

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Abstract

Aberrant activation of the Ras/Raf/ERK (extracellular-signal-regulated kinase)-MAPK (mitogen-activated protein kinase) pathway is involved in the progression of cancer, including urothelial carcinoma; but the negative regulation remains unclear. In the present study, we investigated pathological expression of Spred2 (Sprouty-related EVH1 domain-containing protein 2), a negative regulator of the Ras/Raf/ERK-MAPK pathway, and the relation to ERK activation and Ki67 index in various categories of 275 urothelial tumors obtained from clinical patients. In situ hybridization demonstrated that Spred2 mRNA was highly expressed in high-grade non-invasive papillary urothelial carcinoma (HGPUC), and the expression was decreased in carcinoma in situ (CIS) and infiltrating urothelial carcinoma (IUC). Immunohistochemically, membranous Spred2 expression, important to interact with Ras/Raf, was preferentially found in HGPUC. Interestingly, membranous Spred2 expression was decreased in CIS and IUC relative to HGPUC, while ERK activation and the expression of the cell proliferation marker Ki67 index were increased. HGPUC with membranous Spred2 expression correlated significantly with lower levels of ERK activation and Ki67 index as compared to those with negative Spred2 expression. Thus, our pathological findings suggest that Spred2 counters cancer progression in non-invasive papillary carcinoma possibly through inhibiting the Ras/Raf/ERK-MAPK pathway, but this regulatory mechanism is lost in cancers with high malignancy. Spred2 appears to be a key regulator in the progression of non-invasive bladder carcinoma.

37 # Introduction

38 Bladder cancer is a highly prevalent disease and its incidence is steadily rising worldwide
39 [1]. In the United States, bladder cancer is the 4th most incident and 8th most deadly
40 tumor among men [2]. Most of the bladder cancer is urothelial carcinoma arising from
41 urothelial epithelium. Evidence indicates that urothelial carcinoma has two distinct
42 clinical subtypes with distinct molecular features at bladder tumor initiation; low-grade
43 tumors (superficial papillary) and high-grade tumors (flat, represented by carcinoma in
44 situ) [3, 4]. Low-grade tumors, i.e., papillary urothelial neoplasm of low malignant
45 potential or low-grade papillary urothelial carcinoma, do not easily progress to high-grade
46 papillary urothelial carcinoma or invasive carcinoma [5, 6]. Recently, a comprehensive
47 landscape of molecular alterations in urothelial carcinomas was shown [7]. More than
48 70% of low-grade papillary carcinomas harbor *FGFR3* gene mutation [8]. On the other
49 hand, flat carcinoma in situ (CIS) often develops to invasive urothelial carcinoma [9, 10],
50 in which allelic deletion of the *TP53* (*p53)* and *PTEN* (tumor-suppressor) [11] and
51 *retinoblastoma* gene (RB, negative cell cycle regulator) [12] is common.

52 In addition to the gain of function gene mutations, extracellular-regulated kinase
53 (ERK) plays a crucial role in cancer development and progression [13, 14]. The
54 Ras/Raf/ERK-MAPK (mitogen-activated protein kinase) pathway, one of the
55 serine/threonine kinases of MAPKs pathway, is a major determinant to promote cell
56 proliferation, differentiation, and survival, and plays an important role in bladder cancer
57 prognosis [15]. ERK activation was observed in high-grade non-invasive and invasive
58 urothelial carcinoma [16], suggesting that robust ERK activation contributes to urothelial
59 tumorigenesis with a high malignant potential.

60 Signaling pathways are counterbalanced by endogenous inhibitory mechanism(s).
61 Spred2 (Sprouty-related, EVH1 domain-containing protein 2) inhibits Ras-dependent
62 ERK signaling by suppressing the phosphorylation and activation of Raf [17]. Ras
63 activation is aberrant in many tumors due to oncogenic mutation of the *Ras* genes or
64 alterations in upstream signaling components [18]. Rational therapies that target the
65 Ras/Raf/ERK-MAPK pathway continues to attract much attention for cancer therapy [19].

66 We have hitherto investigated in different types of murine models and found that Spred2
67 controls inflammation by down-regulating the Ras/Raf/ERK-MAPK pathway [20–29].
68 Interestingly, Spred2 expression is down-regulated in invasive carcinomas such as
69 hepatocellular carcinoma [30, 31] and prostatic adenocarcinoma [32]. Thus, altered
70 Spred2 expression could affect urothelial tumorigenesis by regulating the Ras/Raf/ERK-
71 MAPK pathway in bladder cancer. However; the pathophysiological roles of Spred2 in
72 bladder cancer tumorigenesis remain largely unknown. In the present study, we examined
73 the mRNA and protein expression of Spred2 in a range of human urothelial tumors. Our
74 present findings suggest that endogenous Spred2 affects urothelial cancer progression,
75 especially in non-invasive papillary urothelial carcinoma.
76

77 Materials and methods

78 Clinical samples

79 A total of 275 bladder biopsy or resection specimens (transurethral resection and
80 cystectomy) during the year 2001-2016 were retrieved from pathology record at
81 Department of Pathology, Okayama University Hospital. The patients who underwent
82 chemotherapy or radiotherapy before the resection were not included in this study. All the
83 hematoxylin and eosin (HE)-stained sections were reviewed and categorized by two
84 blinded pathologists according to the 2016 WHO classification: non-tumor urothelium
85 (non-tumor), papillary urothelial neoplasm of low malignant potential (PUNLMP), low-
86 grade papillary urothelial carcinoma (LGPUC), high-grade papillary urothelial carcinoma
87 (HGPUC), carcinoma in situ (CIS), and infiltrating urothelial carcinoma (IUC). All
88 sections were used for immunohistochemistry. For in situ hybridization, sections were
89 randomly chosen from each category. Cases for the enrolled 275 patients were shown in
90 Table 1, in which clinicopathological features of each category were noted.
91 The protocol in this study was reviewed and approved by the *Ethics Committee,*
92 *Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical*
93 *Sciences and Okayama University Hospital (1608-009)*. Informed consent was obtained

94 in the form of opt-out on our website. Those who rejected were excluded. This consent
95 procedure conformed to amended Ethical Guidelines for Clinical Studies provided by
96 Ministry of Health, Labor and Welfare of Japan (May 31, 2015) was approved by the
97 *Ethics Committee, Okayama University Graduate School of Medicine, Dentistry and*
98 *Pharmaceutical Sciences and Okayama University Hospital.*
99

100 ## In situ hybridization

101 A total of 85 samples were randomly selected from 275 samples (Table 1). Paraffin-
102 embedded tissue samples were sectioned at 5-μm-thick, kept on glass slides overnight at
103 45°C and then in situ hybridization was performed using the Affymetrix ViewRNA ISH
104 Tissue Assay kit (QVT0050) and ViewRNA Chromogenic Signal Amplification kit
105 (QVT0200) (Thermo Fisher Scientific, MA, USA), according to the manufacturer's
106 instructions. Human Spred2 probe set was purchased from Thermo Fisher (Affymetrix,
107 Catalog No. VA1-17417-01). Spred2 mRNA expression was stained in red-dot. The total
108 number of red-dot in 100 cells was counted in each sample under microscope by two
109 blinded pathologists, and the number of red-dot per cell was calculated.
110

111 ## Immunohistochemistry

112 For immunohistochemistry, all 275 specimens were employed (Table 1). Immunostaining
113 for Spred2 was carried out using the Polink-2 plus HRP rabbit with DAB kit (GBI, Bothell,
114 WA, USA), according to the manufacturer's instructions. In brief, sections (4-μ-thick)
115 were treated by microwave oven in 0.1 M citric acid buffer, treated with 3%H_2O_2 in
116 methanol, blocked with DAKO Protein Block Serum-Free (Dako, Carpinteria, CA, USA),
117 and incubated with anti-human Spred2 polyclonal antibody (Proteintech, Rosemont, IL,
118 USA). Sections were then incubated with rabbit antibody specific enhancer, followed by
119 the addition of polymer-HRP for rabbit IgG, and visualized using DAB complex. Nuclear
120 counterstaining was performed using hematoxylin. Immunostaining for pERK1/2 (Clone
121 D13.14.4E, Cell Signaling Technology, Danvers, MA, USA) and Ki67 (Clone MIB-1,
122 Dako) was performed on a Ventana Discovery XT automated stainer (Ventana, Tucson,

123 AZ, USA) with using iVIEW DAB Detection Kit (Ventana).

124

125 Evaluation of immunohistochemical staining

126 Spred2 was stained in the cytoplasm (C) or/and membrane (M). Immunoreactivity for
127 Spred2 was classified into 4 groups, according to subcellular localization and staining
128 intensity; C-M-, absent or weak staining intensity in cytoplasm and membrane; C-M+,
129 moderate to strong membranous staining without staining in cytoplasm; C+M-, moderate
130 to strong cytoplasmic staining without membranous staining; C+M+, moderate to strong
131 cytoplasmic and membranous staining. pERK immunostaining was scored on the
132 following semiquantitative scale as previously reported with modifications [33]: no
133 staining (0); focal to <10% of cells (1); 10-50% of cells (2); 50% or more cells stained
134 weak (3); 10-50% stained strong (4); 50% or more stained strong (5). Ki67 index, a
135 marker for cell proliferation, was determined by counting 500 tumor cells, and the
136 percentages of positively stained cells were determined. The stained sections were
137 assessed by two blinded pathologists.

138

139 Database analysis

140 Datasets with more than 25 samples in each category from Sanchez-Carbayo bladder 2
141 [34], Blaveri bladder 2 [35], and Stransky bladder [36] were used to analyze Spred2
142 expression in bladder cancer. An unpaired two-tailed t test was used for the statistical
143 analysis. Kaplan-Meier Plotter (http://www.kmplot.com) was used to analyze the
144 prognostic values of *Spred2* mRNA expression levels in bladder carcinoma. Kaplan-
145 Meier survival plots were drawn using data from the Kaplan-Meier database. A log-rank
146 *p*-value <0.05 was considered to indicate a statistically significant difference.

147

148 Statistical analysis

149 Statistical analysis was performed using GraphPad Prism7 (GraphPad Software, San
150 Diego, CA, USA) and js-STAR (free software). Dunn's multiple comparison test was
151 performed after Kruskal-Wallis test for the comparison of mean values among multi-

152 groups. Multiple Fisher's exact test was performed using the Bonferroni correction for the
153 comparison of proportions among multi-groups. Mann-Whitney test was used for the
154 comparison of mean values between the two groups. A value of $p<0.05$ was considered
155 statistically significant.
156

157 Results

158 *Spred2* mRNA expression in bladder urothelial tumors

159 We first examined *Spred2* mRNA expression in various categories of 85 urothelial lesions
160 including non-tumor, PUNLMP, LGPUC, HGPUC, CIS, and IUC. Figure 1A shows the
161 representative HE and in situ hybridization photographs from each category, in which
162 *Spred2* mRNA expression was presented by red-dot (Fig 1A). The number of red-dots per
163 cell was regarded as *Spred2* mRNA expression level (Fig 1B). Levels of *Spred2* mRNA
164 expression were increased as the malignancy of the cancer increased in papillary tumors.
165 Of note, the level reached the peak in HGPUC and then decreased in CIS and IUC. *Spred2*
166 mRNA expression in IUC was significantly lower than that in HGPUC (Fig 1B). These
167 results indicate that *Spred2* mRNA expression was up-regulated in non-invasive papillary
168 bladder cancer as compared to cancers with high malignancy including invasive
169 carcinoma.
170

171 Spred2 protein expression in bladder urothelial tumors

172 We next examined Spred2 protein expression by immunohistochemistry in 275 bladder
173 urothelial tumors. To confirm immunoreactivity of Spred2 antibody, H1993 cells were
174 stained with the antibody under overexpressing Spred2 (S1 Fig). Spred2 protein
175 expression (Fig 2A) was immunophenotypically classified into 4 groups, according to the
176 subcellular localization and staining intensity. The staining pattern in each tumor category
177 was shown in Table 2. In all non-tumor cases, Spred2 was positive in cytoplasm of basal
178 and lower intermediate cells (pattern C+M-, 101/101 cases). More than half of the cases
179 of PUNLMP, CIS, and IUC showed absent or weak staining (C-M-; 74% (14/19 cases),
180 74% (29/39 cases), and 69% (22/32 cases), respectively). LGPUC and HGPUC showed

membranous staining (C-M+ and C+M+) more frequently (49% (20/41 cases), and 51% (28/43 cases), respectively) than other categories (Table 2). We then compared mRNA and protein expression of Spred2. Cases with membranous staining, regardless of cytoplasmic staining pattern (C^-M^+ and C^+M^+), showed significantly higher levels of *Spred2* mRNA expression than those without membranous staining (C^-M^- and C^+M^-) (Fig 2B). Spred2 is a membrane-associated substrate of receptor tyrosine kinase [17, 37] and react with Raf localized in the raft domain of the plasma membrane [38], suggesting that membranous Spred2 is more meaningful when considering the functional regulation. The positive rate of membranous Spred2 expression (C^-M^+ and C^+M^+) in each category was shown in Figure 2C, which showed that the expression was increased in LGPUC, peaked in HGPUC and then decreased in CIS and IUC. Together with the mRNA expression data, these results suggest that functional Spred2 was most highly expressed in HGPUC, and the expression was lower in CIS and IUC as compared to HGPUC.

Expression of pERK and Ki67 in bladder urothelial tumors

Increased Spred2 expression may affect the activation of the Ras/Raf/ERK-MAPK pathway and subsequent cancer growth. To address this possibility, we investigated the protein expression of phosphorylated ERK (pERK), an indicator of ERK-MAPK activation status, immunohistochemically in each category. pERK was detected in the nucleus and cytoplasm of urothelial epithelial lesions in all specimens from each category with different intensity in strength (Fig 3A). The intensity of nuclear and cytoplasmic staining of pERK was then evaluated, which showed that pERK score in each category increased with increasing malignant potential (Fig 3B). Table 3 summarized pERK score in each Spred2 immunostaining pattern. Weak pERK staining (score, 1 and 2) was detected in 87% (score 1; 67/101, score 2; 21/101 cases) and 100% (score 1; 13/19, score 2; 6/19 cases) of non-tumor and PUNLMP, respectively. In cancer categories (LGPUC, HGPUC, CIS and IUC), the number of cases with moderate (score 3) and strong (score 4 and 5) staining increased. Strong pERK staining was detected in 10% (score 4; 4/41, score 5; 0/41), 44% (score 4; 14/43, score 5; 5/43 cases), 56% (score 4; 14/39, score 5; 8/39

210 cases), and 78% (score 4; 16/32, score 5; 9/32 cases) in LGPUC, HGPUC, CIS and IUC,
211 respectively (Table 3). Figure 3C demonstrated the relation between Spred2
212 immunostaining pattern and pERK score for all cases in each category. We next
213 performed Ki67 immunostaining (Fig 4A) and calculated Ki67 index (Fig 4B), an
214 indicator of cell proliferation marker, in all categories. Ki67 index was significantly
215 increased in all categories of bladder urothelial tumors as compared to non-tumor. Ki67
216 index of HGPUC, CIS and IUC was significantly higher than that of LGPUC (Fig 4B).
217 Figure 4C demonstrated the relation between pERK and Ki67 index for all cases in this
218 study. Thus, pERK score and Ki67 index increase with increasing malignancy.
219

220 Correlation between pERK score/Ki67 index and membranous 221 Spred2 expression

222 We next compared the correlation between pERK score/Ki67 index and membranous
223 Spred2 expression (negative: M-, positive: M+) in cancer categories. In HGPUC, pERK
224 score with Spred2 M+ were lower than those with Spred2 M-. No differences were found
225 in LGPUC, CIS and IUC (Fig 5A). Since an increase in pERK score is generally
226 associated with an increased Ki67 index [39], ERK activation may result in increased
227 tumor cell proliferation. As shown in Figure 5B, Ki67 index in HGPUC with Spred2 M+
228 was lower than those with Spred2 M-. These results suggest that membranous Spred2
229 plays a role in down-regulated ERK activation and subsequent cancer cell proliferation
230 in HGPUC, but this negative regulatory mechanism is not functioning in CIS. Although
231 pERK score was not different between Spred2 M- and Spred2 M+ in IUC, Ki67 index
232 was decreased in Spred2 M+ as compared to Spred2 M-, suggesting that Spred2 may
233 downregulate cancer cell proliferation through a mechanism independent of ERK-MAPK
234 in IUC.
235

236 Database analyses of Spred2 expression and overall survival

237 We examined *Spred2* mRNA expression in bladder cancer database by Sanchez-Carbayo
238 bladder 2 dataset [34], Blaveri bladder 2 [35], and Stransky bladder [36] in a public cancer

239 microarray database (ONCOMINE) [40]. As shown in Figure 6A, *Spred2* mRNA
240 expression was significantly increased in non-invasive superficial bladder cancer
241 compared to that in normal bladder samples (Fig 6A, left). Of note, *Spred2* mRNA
242 expression in infiltrating bladder urothelial carcinoma was lower than that in superficial
243 bladder cancer in all three datasets (Fig 6A, middle and right). These data were consistent
244 with that in Figure 1B, which showed that *Spred2* mRNA expression was upregulated in
245 non-invasive papillary bladder cancer as compared to invasive carcinoma. The decreased
246 *Spred2* mRNA expression in infiltrating bladder urothelial carcinoma may have affected
247 cancer survival. We then assessed the prognostic value of Spred2 in patients with bladder
248 carcinoma by Kaplan-Meier Plotter (www.kmplot.com). The overall survival for 30
249 months was higher in patients with higher *Spred2* mRNA level (Fig 6B). Although there
250 was no statistical significance in the 150 month-overall survival between the groups (Fig
251 6C, upper panel), the median survival in high *Spred2* mRNA expression group (42.33
252 months) was 1.6 times longer than low expression group (28.63 months) (Fig 6C, lower
253 panel). Thus, the expression level of Spred2 mRNA can be clinically important for the
254 prognosis of bladder cancer patients.

255

256 Discussion

257 Cancer cell growth is mediated by various cell signaling pathways. Among them, the
258 Ras/Raf/ERK-MAPK pathway is often activated in human diseases including cancer [41],
259 and as such represents an attractive target for the development of anti-cancer drugs [19].
260 This pathway is also important in urothelial cell migration and invasion [42]. A better
261 understanding of the endogenous negative regulatory mechanism(s) could improve
262 strategies for preventing and treating bladder urothelial tumors. To the best of our
263 knowledge, this is the first report to show Spred2 mRNA and protein expression in
264 bladder urothelial tumors in all categories, ranging from non-tumor to invasive cancer.

265 Previous studies demonstrated that *Spred2* mRNA expression was decreased in
266 hepatocellular carcinoma (HCC) [31] and prostatic adenocarcinoma [32], comparing with
267 that in adjacent non-tumor tissue and benign gland, respectively. Down-regulated Spred2

268 expression was particularly evident in higher grade prostate cancers [32], and Spred2
269 expression levels in HCC tissue were inversely correlated with the incidence of tumor
270 invasion and metastasis [31]. These previous findings suggested that Spred2 function as
271 a potential tumor suppressor gene. In our study, *Spred2* mRNA expression was increased
272 in non-invasive cancer HGPUC, whereas the expression in invasive bladder cancer IUC
273 was significantly decreased as compared to that in non-invasive carcinoma HGPUC.
274 Consistently, database analyses showed that Spred2 expression in infiltrating bladder
275 urothelial carcinoma (invasive) was lower than that in superficial bladder cancer (non-
276 invasive). Protein expression of functional Spred2, a membranous positive staining, was
277 frequently observed in LGPUC and HGPUC, but not in CIS and IUC. Thus, Spred2 may
278 play a role as a tumor suppressor in non-invasive carcinomas, but the function appears to
279 be lost in invasive carcinoma.

280 Spred2 was discovered as a membrane-associated substrates of receptor tyrosine
281 kinases [17, 37]. However; our data indicated that Spred2 was found not only in the
282 membrane but in the cytoplasm in urothelial tumors. Previous confocal microscopy
283 analyses revealed that Spred2 was present in cytoplasm and co-localized with neighbor
284 of BRCA1 (NBR1) [43] or microtubule-associated protein 1A/1B-light chain 3
285 (*LC3*) [44]. Very interestingly, Spred2-NBR1 complex enhanced Spred2-mediated ERK
286 inhibition upon stimulation with fibroblast growth factor (FGF), suggesting that
287 Spred2/NBR1-dependent down-regulation of ERK-MAPK is achieved via directed
288 endosomal trafficking of activated receptors [43]. Sprouty proteins, members of the
289 Sprouty/Spred family, were distributed throughout the cytosol, which underwent rapid
290 translocation to membrane ruffles following epidermal growth factor (EGF) stimulation
291 [45]. In urothelial tumors, we showed that membranous Spred2 protein was often detected
292 in cancer categories, especially LGPUC and HGPUC. These results suggest that Spred2
293 translocates from cytoplasm to cell membrane by various stimuli in the cancer
294 microenvironment, exerting its function as a negative regulator of ERK-MAPK.

295 ERK activation was associated with increased Ki67 expression in salivary gland
296 mucoepidermoid carcinoma [39]. Since Spred2 inhibits the ERK pathway and subsequent

297 cell proliferation, we compared the relationship between membranous Spred2 protein
298 expression and pERK score/Ki67 index in each cancer category. Interestingly, HGPUC
299 displaying membranous Spred2 expression showed significantly lower pERK score and
300 Ki67 index, as compared to other categories with membrane-negative expression. On the
301 other hand, pERK score was not affected by the expression pattern of Spred2 expression
302 in CIS and IUC. Spred2 is presumed to be effective only after reaching a certain level of
303 membrane expression. It appears that ERK activation was so strong in CIS and IUC that
304 concurrent membranous Spred2 expression might be insufficient to suppress the aberrant
305 ERK activation in CIS and IUC. *Spred2* gene mutations can be frequently seen in bladder
306 urothelial carcinoma (S2 Fig). Spred2 in CIS and IUC can be mutated, which may fail to
307 modulate Ras-Raf interaction. Alternatively, mutational activation of the MAP kinase
308 pathway including Ras/Raf is occasionally found in many types of cancer including
309 urothelial carcinoma [46, 47]. Spred2 suppresses Ras signaling by preventing
310 the phosphorylation and activation of Raf [17]. Mutated Ras/Raf may fail to interact with
311 Spred2. Interestingly, Ki67 index was decreased in IUC with membranous Spred2
312 expression, although pERK score was not altered by membranous Spred2 expression.
313 Spred2 may downregulate cancer cell proliferation through an ERK-MAPK independent
314 pathway in IUC.

315 *Spred2* mRNA expression in CIS was as high as that in HGPUC, however; 75% of
316 CIS showed negative membranous Spred2 staining and only 15% of CIS showed positive
317 membranous Spred2 staining. It remains unclear how Spred2 protein expression is
318 regulated in CIS. The poor correlations were generally reported between the level of
319 mRNA and protein [48, 49]. There are many complicated and varied post-transcriptional
320 mechanisms; post-transcriptional, translational and protein degradation regulation. CIS
321 appears to be the critical turning-point to control the complex regulation. Further study is
322 necessary to understand the specific mechanisms regulating Spred2 mRNA and protein
323 expression.

324 In conclusion, Spred2 mRNA and protein expression was up-regulated as the grade
325 increased in non-invasive papillary urothelial carcinomas. Membranous Spred2

expression in HGPUC, but not in CIS and IUC, correlated significantly with low levels of ERK activity. In bladder cancer, HGPUC is clinically important because tumor grows more quickly and more likely spread, and tumor progression (invasion) was identified in 40% of all cases [50]. Our present study suggests that Spred2 functions to suppress the growth and progression of cancer in non-invasive bladder cancer through suppressing the ERK pathway, and this regulatory mechanism no longer functions in invasive bladder cancer.

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497

Supporting information

S1 Fig. Overexpression of Spred2 in H1993 cells. H1993 cells were transfected with Spred2 expression plasmid (OriGene, Rockville, MD, USA) or control plasmid (OriGene) using turbofectin 8.0 (OriGene). (A) *Spred2* mRNA expressions after transfection with control plasmid (control) or Spred2 expression plasmid (Spred2) were analyzed by RT-qPCR (n=3 each). Data is presented as mean ± SEM. $^{*}p<0.0001$ (unpaired t test). (B) Transfection was carried out on Lab-Tek II Slide (8 Chamber, Electron Microscopy Sciences, Hatfield, PA, USA). The cells were fixed in 95% ethanol and immunostained with anti-Spred2 polyclonal antibody using the polymer method (Polink-2 Plus HRP RABBIT with DAB kit, GBI, Bothell, WA, USA). Spred2 positive cells were shown in brown. (C) Cell extracts after transfection with control plasmid (control) or Spred2 expression plasmid (Spred2) were immunoblotted with indicated primary antibodies (n=3 each). Upper: Representative photos were shown. Lower: Band densities were digitised and semi-quantitated. Data is presented as mean ± SEM. (D) Ki67 mRNA expressions after transfection with control plasmid (control) or Spred2 expression plasmid (Spred2) were analyzed by RT-qPCR (n=3 each). Data is presented as mean ± SEM. $^{\#}p<0.05$ (unpaired t test). (E) After transfection with control plasmid (control) or Spred2 expression plasmid (Spred2), the cell proliferation was evaluated by CCK-8 assay (n=3 each). Data is presented as mean ± SE. $^{*}p<0.0001$ (unpaired t test).

S2 Fig. The mutation of Spred2 in cancers. Data were from TCGA Pancancer Atlas from cBioPortal for Cancer Genomics (https://www.cbioportal.org/results/plots). (A) The Spred2 mutations in different cancer. Bladder cancer is the 3rd place having mutation of Spred2 among cancers. (B) The distribution of mutations on the domain structure of Spred2 in bladder urothelial carcinoma.

524 # Figure legends

525

526 **Fig 1. Spred2 mRNA expression in urothelial tumors.** (A) Representative photographs
527 of HE- (original magnification 400×) and in situ hybridization-sections from each
528 category are shown. Spred2 mRNA expression was presented by red dots. (B) The number
529 of the red-dots per cell was counted under microscope and Spred2 mRNA expression
530 level was shown per one cell from each category (N: non-tumor; n=10, P: PUNLMP;
531 n=10, L: LGPUC; n=15, H: HGPUC; n=18, C: CIS; n=18, and I: IUC; n=14). Data were
532 mean ± SEM. $^{\#}p<0.05$, $^{\S}p<0.01$, $^{\P}p<0.001$, $^{*}p<0.0001$ (Dunn's multiple comparison test).

533

534 **Fig 2. Immunohistochemical analyses of Spred2 protein expression in urothelial**
535 **tumors.** (A) Representative photographs of Spred2 immunohistochemistry (original
536 magnification 400×) from each category are shown. (B) Expression levels of Spred2
537 mRNA in each Spred2 staining pattern were shown. C; cytoplasm, M; membrane. (C-M-;
538 n=81, C+M-; n=122, C-M+; n=54, C+M+; n=11). Data were mean ± SEM. $^{\#}p<0.05$,
539 $^{\S}p<0.01$ (Dunn's multiple comparison test). (C) The positive rate of membranous Spred2
540 expression in each category was shown (N: non-tumor; n=101, P: PUNLMP; n=19, L:
541 LGPUC; n=41, H: HGPUC; n=43, C: CIS; n=39, and I: IUC; n=32). Data were mean ±
542 SEM. $^{\#}p<0.05$, $^{\S}p<0.01$ (Multiple Fisher's exact test).

543

544 **Fig 3. pERK score in urothelial tumors.** (A) Representative photographs of pERK
545 immunohistochemistry (original magnification 400×) from each category are shown. (B)
546 pERK staining intensity was evaluated and scored (0-5), and pERK score in each category
547 was shown (N: non-tumor; n=101, P: PUNLMP; n=19, L: LGPUC; n=41, H: HGPUC;
548 n=43, C: CIS; n=39, and I: IUC; n=32). Data were mean ± SEM. $^{\S}p<0.01$, $^{\P}p<0.001$,
549 $^{*}p<0.0001$ (Dunn's multiple comparison test).

550

551 **Fig 4. Ki67 index in urothelial tumors.** (A) Representative photographs of Ki67
552 immunohistochemistry (original magnification 400×) from each category are shown. (B)

553 Ki67 index in each category was shown (N: non-tumor; n=101, P: PUNLMP; n=19, L:
554 LGPUC; n=41, H: HGPUC; n=43, C: CIS; n=39, and I: IUC; n=32). Data were mean ±
555 SEM. $^{\#}p<0.05$, $^{\S}p<0.01$, $^{\P}p<0.001$, $^{*}p<0.0001$ (Dunn's multiple comparison test).

556

557 **Figure 5. Comparison between pERK score/Ki67 index and membranous Spred2**
558 **expression.** pERK score (A) and Ki67 index (B) were compared between membranous
559 Spred2 negative (M-) and positive (M+) in cancer categories (LGPUC; n=41 (M-:21,
560 M+:20), HGPUC; n=43 (M-:15, M+:28), CIS; n=39 (N-:33, M+:6), and IUC; n=32
561 (M-:22, M+:10)). Bar in each graph represents median. $^{\#}p<0.05$, $^{\S}p<0.01$ (Mann-Whitney
562 test).

563

564 **Fig 6. Spred2 expression in overall survival of patients with bladder cancer.** (A)
565 Statistical analyses of Spred2 expression in normal, superficial bladder cancer
566 (superficial) and infiltrating bladder urothelial carcinoma (infiltrating) from 3 different
567 datasets (Sanchez-Carbayo bladder 2, Blaveri bladder 2, and Stransky bladder) were
568 shown. The numbers in parentheses indicates the number of samples. $^{\S}p<0.01$, $^{*}p<0.0001$
569 (unpaired two-tailed t test). (B, C) Kaplan-Meier analysis of the data in www.kmplot.com
570 was used to determine the survival probability for 30 months (B) and 150 months (C) of
571 patients with high or low Spred2 expression, followed by the log-rank test.
572

Table 1. Cases for the enrolled 275 patients.

	cases (%)	number of examined by IHC/ISH	features		
			progression	nuclear grade	invasiveness
non-tumor	101 (36.7)	101/10	-	-	-
PUNLMP	19 (6.9)	19/10	slow	very low	non-invasive
LGPUC	41 (14.9)	41/15	slow	low	non-invasive
HGPUC	43 (15.6)	43/18	quick	high	non-invasive
CIS	39 (14.2)	39/18	quick	high	non-invasive
IUC	32 (11.6)	32/14	quick	high>>low	invasive

non-tumor; non-tumor urothelium, PUNLMP; papillary urothelial neoplasm of low malignant potential, LGPUC; low-grade papillary urothelial carcinoma, HGPUC; high-grade urothelial carcinoma, CIS; carcinoma in situ, IUC; infiltrating urothelial carcinoma. IHC; immunohistochemistry, ISH; in situ hybridization.

579 **Table 2. Subcellular immunolocalization of Spred2 in each tumor category**

	C-M-	C+M-	C-M+	C+M+	total cases
non-tumor	0	101	0	0	101
PUNLMP	14	4	1	0	19
LGPUC	15	6	20	0	41
HGPUC	8	7	22	6	43
CIS	29	4	4	2	39
IUC	22	0	7	3	32
total cases	88	122	54	11	275

580 non-tumor; non-tumor urothelium, PUNLMP; papillary urothelial neoplasm of low malignant
581 potential, LGPUC; low-grade papillary urothelial carcinoma, HGPUC; high-grade urothelial
582 carcinoma, CIS; carcinoma in situ, IUC; infiltrating urothelial carcinoma. IHC;
583 immunohistochemistry, ISH; in situ hybridization. C; cytoplasm, M; membrane.
584

585 **Table 3. pERK score in Spred2 immunostaining pattern**

Spred2 pattern \ pERK score		1	2	3	4	5	total cases
non-tumor	C-M-	0	0	0	0	0	0
	C+M-	67	21	13	0	0	101
	C-M+	0	0	0	0	0	0
	C+M+	0	0	0	0	0	0
PUNLMP	C-M-	11	3	0	0	0	14
	C+M-	2	2	0	0	0	4
	C-M+	0	1	0	0	0	1
	C+M+	0	0	0	0	0	0
LGPUC	C-M-	3	4	8	0	0	15
	C+M-	1	0	3	2	0	6
	C-M+	0	5	13	2	0	20
	C+M+	0	0	0	0	0	0
HGPUC	C-M-	0	1	2	4	1	8
	C+M-	0	0	2	3	2	7
	C-M+	0	4	14	4	0	22
	C+M+	0	0	1	3	2	6
CIS	C-M-	0	1	11	11	6	29
	C+M-	0	0	2	1	1	4
	C-M+	0	0	2	1	1	4
	C+M+	0	0	1	1	0	2
IUC	C-M-	0	0	4	11	7	22
	C+M-	0	0	0	0	0	0
	C-M+	0	1	1	3	2	7
	C+M+	0	0	1	2	0	3

586 non-tumor; non-tumor urothelium, PUNLMP; papillary urothelial neoplasm of low malignant
587 potential, LGPUC; low-grade papillary urothelial carcinoma, HGPUC; high-grade urothelial
588 carcinoma, CIS; carcinoma in situ, IUC; infiltrating urothelial carcinoma. IHC;
589 immunohistochemistry, ISH; in situ hybridization.