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Shinsuke Oda
Okayama University
Masayoshi Fujisawa
Okayama University
Li Chunning
Okayama University
Toshihiro Ito
Nara Medical University
Takahiro Yamaguchi
Okayama University
Teizo Yoshimura
Okayama University
Akihiro Matsukawa (amatsu@md.okayama-u.ac.jp)
Okayama University

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Expression of Spred2 in the urothelial tumorigenesis of the urinary bladder

Shinsuke Oda[1], Masayoshi Fujisawa[1], Li Chunning[1], Toshihiro Ito[2], Takahiro Yamaguchi[1], Teizo Yoshimura[1], and Akihiro Matsukawa[1]*

[1]Department of Pathology and Experimental Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, 700-8558, Japan.
[2]Department of Immunology, Nara Medical University, Kashihara, 634-8521, Japan.

*Corresponding author**:**
Akihiro Matsukawa, M.D., Ph.D.
Department of Pathology and Experimental Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University
2-5-1 Shikata, Kita-ku, Okayama 700-8558, Japan
TEL +81-86-235-7141
FAX +81-86-235-7148
E-mail: amatsu@md.okayama-u.ac.jp

Abstract

Aberrant activation of the Ras/Raf/ERK-MAPK pathway is involved in the progression of cancer, including urothelial carcinoma; but the negative regulation remains unclear. Here, 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 a cell proliferation marker 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 carcinoma in situ (CIS), and the expression was decreased in invasive 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 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 negatively regulates cancer progression in non-invasive papillary carcinoma possibly through inhibiting Ras/Raf/ERK-MAPK pathway, but this regulatory mechanism is lost in CIS and IUC. Spred2 appears to be a key regulator in the progression of non-invasive bladder carcinoma.

Introduction

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

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

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

inflammation by down-regulating the Ras/Raf/ERK-MAPK pathway[20–29]. Interestingly, Spred2 expression is down-regulated in invasive carcinomas such as hepatocellular carcinoma[30,31] and prostatic adenocarcinoma[32]. Thus, altered Spred2 expression could affect urothelial tumorigenesis by regulating the Ras/Raf/ERK-MAPK signaling in bladder cancer. However; the pathophysiological roles of Spred2 in bladder cancer tumorigenesis remain largely unknown. In the present study, we examined the mRNA and protein expression of Spred2 in a range of human urothelial tumors. Our present findings suggest that endogenous Spred2 affects urothelial cancer progression, especially in non-invasive status.

Results

Spred2 mRNA expression in bladder urothelial tumors

We first examined Spred2 mRNA expression in various categories of 85 urothelial lesions including normal urothelium (NORMAL), papillary urothelial neoplasm of low malignant potential (PUNLMP), low-grade papillary urothelial carcinoma (LGPUC), high-grade papillary urothelial carcinoma (HGPUC), carcinoma in situ (CIS), and invasive urothelial carcinoma (IUC) (Table 1). Fig. 1a shows the representative HE and in situ hybridization photographs from each category, in which Spred2 mRNA expression was presented by red-dot (Fig. 1a). The number of red-dots per cell was regarded as Spred2 mRNA expression level (Fig. 1b). Levels of Spred2 mRNA expression were increased as the malignancy of the cancer increased in papillary tumors. Of note, the level reached the peak in HGPUC and then decreased in CIS and IUC. Spred2 mRNA expression in IUC was significantly lower than that in HGPUC (Fig. 1b). These results indicate that Spred2 mRNA expression was up-regulated in non-invasive bladder cancer as compared to invasive carcinoma.

Spred2 protein expression in bladder urothelial tumors

We next examined Spred2 protein expression by immunohistochemistry in 275 bladder urothelial tumors. To confirm immunoreactivity of Spred2 antibody, H1993 cells were

stained with the antibody under overexpressing Spred2 (Supplementary Fig. 1). Spred2 protein expression (Fig. 2a) was immunophenotypically classified into 4 groups, according to the subcellular localization and staining intensity (Table 2). The proportion of staining pattern within each category was shown in Fig. 2b. In all NORMAL cases, Spred2 was positive in cytoplasm of basal and lower intermediate cells (pattern C+M-, 101 cases). More than half of the cases of PUNLMP, CIS, and IUC showed absent or weak staining (C-M-; 74% (14/19 cases), 74% (29/39 cases), and 69% (22/32 cases), respectively) (Fig. 2b). LGPUC and HGPUC showed membranous staining (C-M+ and C+M+) more frequently (49% (20/41 cases), and 51% (22/43 cases) + 14% (6/43 cases), respectively) than other categories (Fig. 2b). 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. 2c). Spred2 is a membrane-associated substrate of receptor tyrosine kinase[33] and react with Raf localized in the raft domain of the plasma membrane[34]. Thus, membranous Spred2 appears to be more meaningful when considering the functional regulation. Together with the mRNA expression data, these results suggest that Spred2 was most expressed in HGPUC, and the expression was decreased in CIS and IUC.

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, by 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 was evaluated. Weak pERK staining (score, 1 and 2) was detected in 87% (score 1; 67/101, score 2; 21/101 cases) and 100% (score 1; 14/19, score 2; 5/19 cases) of NORMAL and PUNLMP, respectively (Fig. 3b). In cancer categories (LGPUC, HGPUC,

CIS and IUC), cancer cells with moderate (score 3) and strong (score 4 and 5) staining were increased. Strong pERK staining was detected in 47% (score 4; 15/43, score 5; 5/43 cases), 59% (score 4; 14/39, score 5; 9/39 cases), and 78% (score 4; 16/32, score 5; 9/32 cases), of HGPUC, CIS and IUC, respectively. We next performed Ki67 immunohistochemistry (Fig. 4a) and calculated Ki67 index (Fig. 4b), an indicator of cell proliferation marker. Ki67 index was significantly increased in all categories of bladder urothelial tumors as compared to NORMAL. Ki67 index of HGPUC, CIS and IUC was significantly higher than that of LGPUC (Fig. 4b). Thus, pERK score and Ki67 index increase as the malignancy of the cancer increases.

Comparison between Spred2 expression and pERK score/Ki67 index.

Spred2 expression and pERK score/Ki67 index are expected to have the opposite relationship. To confirm this, we compared Spred2 expression pattern and pERK score in LGPUC, HGPUC, CIS, and IUC (Fig. 5). In HGPUC, pERK score in Spred2 C-M+ pattern was significantly lower than that in Spred2 C+M- pattern. Spred2 staining pattern did not correlate with pERK score in LGPUC, CIN, and IUC (Fig. 5). We then compared Spred2 expression pattern and Ki67 index in LGPUC, HGPUC, CIS, and IUC (Fig. 6). There were no applicable cases of Spred2 C+M+ pattern and C+M- pattern in LGPUC and IUC, respectively. In HGPUC, Ki67 index in Spred2 C-M+ pattern was significantly lower than that in Spred2 C-M- pattern. Since an increase in pERK is generally associated with an increased Ki67 index[35], ERK pathway activation results in increased tumor cell proliferation. These results suggest that membranous Spred2 plays a role in down-regulated ERK activation and subsequent cancer cell proliferation in HGPUC, but this negative regulatory mechanism is not functioning in CIS and IUC.

Database analyses of Spred2 expression and overall survival.

We examined Spred2 expression in bladder cancer database by Sanchez-Carbayo bladder 2 dataset[36], Blaveri bladder 2[37], and Stransky bladder[38] in a public cancer microarray database (ONCOMINE)[39]. As shown in Fig. 7a, Spred2 expression was significantly

increased in non-invasive superficial bladder cancer compared to that in normal bladder samples (Fig. 7a, left). Of note, Spred2 expression in infiltrating (invasive) bladder urothelial carcinoma was lower than superficial bladder cancer, which was also found in the other datasets (Fig. 7a, middle and right). The decreased Spred2 expression in infiltrating bladder urothelial carcinoma may have affected cancer survival. We then assessed the prognostic value of Spred2 in patients with bladder carcinoma in Kaplan-Meier Plotter (www.kmplot.com). The overall survival for 30 months was higher in patients with higher Spred2 mRNA level (Fig. 7b). Although there was no statistical significance in the 150 month-overall survival between the groups (Fig. 7c, upper panel), the median survival in Spred2 high expression cohort (42.33 months) was 1.6 times longer than low expression cohort (28.63 months) (Fig. 7c, lower panel). Thus, the expression level of Spred2 can be clinically important in the cancer progression.

Discussion

Cancer cell growth is mediated by various cell signaling pathways. Among them, Ras/Raf/ERK-MAPK is often up-regulated in human diseases including cancer[40], and as such represents an attractive target for the development of anti-cancer drugs[19]. This pathway is also important in urothelial cell migration and invasion[41]. A better understanding of the endogenous negative regulatory mechanism(s) could improve strategies for preventing and treating bladder urothelial tumors. To the best of our knowledge, this is the first report to show Spred2 mRNA and protein expression in bladder urothelial tumors in all categories, ranging from normal to invasive cancer.

Previous studies demonstrated that Spred2 mRNA expression was decreased in hepatocellular carcinoma (HCC)[31] and prostatic adenocarcinoma[32], comparing with that in adjacent non-tumor tissue and benign gland, respectively. Down-regulated Spred2 expression was particularly evident in higher grade prostate cancers[32], and Spred2 expression levels in HCC tissue were inversely correlated with the incidence of tumor invasion and metastasis[31]. These previous findings suggested that Spred2 may function as a potential tumor suppressor gene. Similarly, in our study Spred2 mRNA expression in

invasive bladder cancer IUC was significantly decreased as compared to that in non-invasive carcinoma HGPUC, but Spred2 expression was increased in non-invasive cancers. Consistently, database analyses showed that Spred2 expression in infiltrating bladder urothelial carcinoma (invasive) was lower than that in superficial bladder cancer (non-invasive). Immunohistochemically, urothelial tumors with Spred2 membranous expression harbored significantly higher levels of Spred2 mRNA expression than those with cytoplasmic localization and negative staining. A previous study showed that Spred2 colocalizes with Ras and suppresses the phosphorylation and activation of Raf, thereby inhibits subsequent activation of ERK-MAPK[17], suggesting that the membranous Spred2 in urothelial tumors is functionally important. The membranous expression was frequently observed in LGPUC and HGPUC, thus Spred2 appeared to be most active in these tumors. Thus, Spred2 may play a role as a tumor suppressor in non-invasive carcinomas, but the function appears to be lost in invasive carcinomas.

Spred2 mRNA expression in CIS was as high as that in HGPUC, however; 75% of CIS showed negative Spred2 staining and only 15% of CIS showed positive membranous Spred2 staining. It remains unclear how Spred2 protein expression is regulated in CIS. The poor correlations were generally reported between the level of mRNA and protein[42,43]. There are many complicated and varied post-transcriptional mechanisms; post-transcriptional, translational and protein degradation regulation. CIS appears to be the critical turning-point to control the complex regulation. Further study is necessary to understand the specific mechanisms regulating Spred2 mRNA and protein expression.

ERK activation was associated with increased Ki67 expression in salivary gland mucoepidermoid carcinoma[35]. Since Spred2 inhibits the ERK pathway and subsequent cell proliferation, we compared the relationship between Spred2 expression pattern and pERK score/Ki67 index in each tumor category. As expected, HGPUC displaying Spred2 membranous staining without cytoplasmic staining showed significantly lower pERK score and Ki67 index. However, high levels of pERK score/Ki67 index were observed in HGPUC with concurrent membranous and cytoplasmic Spred2 staining. As described above, Spred2 is presumed to be effective only after reaching a certain level of membrane-

restricted expression. Concurrent membranous and cytoplasmic expression of Spred2 might be insufficient to suppress ERK activation. On the other hand, pERK score/Ki67 index was not affected by membranous Spred2 expression in CIS and IUC. It appears that ERK activation was so strong that membranous Spred2 fails to suppress ERK-MAPK pathway in CIS and IUC. Alternatively, Spred2 gene mutations can be frequently seen in bladder urothelial carcinoma (Supplementary Fig. 2). The mutated Spred2 may no longer function normally.

In summary, Spred2 mRNA and protein expression was up-regulated as the grade increased in non-invasive papillary urothelial carcinomas. Membrane-restricted Spred2 expression in high-grade non-invasive papillary urothelial carcinoma, but not in CIS and IUC, correlated with significantly low levels of ERK activity and cell proliferation. In bladder cancer, high-grade non-invasive papillary urothelial carcinoma is clinically important because tumor grows more quickly and more likely spread, and tumor progression (invasion) was identified in 40% of all cases[44]. 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 does not function in invasive bladder cancer.

Methods

Clinical samples

A total of 275 bladder biopsy or resection specimens (transurethral resection and cystectomy) during the year 2001-2016 were retrieved from pathology record at Department of Pathology, Okayama University Hospital. The patients who underwent chemotherapy or radiotherapy before the resection were not included in this study. All the hematoxylin and eosin (HE)-stained sections were reviewed and categorized by two blinded pathologists according to the 2016 WHO classification: normal urothelium (NORMAL), papillary urothelial neoplasm of low malignant potential (PUNLMP), low-grade papillary urothelial carcinoma (LGPUC), high-grade papillary urothelial carcinoma (HGPUC), carcinoma in situ (CIS), and invasive urothelial carcinoma (IUC). All sections

were used for immunohistochemistry. For in situ hybridization, sections were randomly chosen from each category. Cases for the enrolled 275 patients were shown in Table 1, in which clinicopathological features of each category were noted.

The protocol in this study was reviewed and approved by the *Ethics Committee*, *Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Okayama University Hospital (1608-009)*. Informed consent was obtained in the form of opt-out on out web-site. Those who rejected were excluded. This consent procedure conformed to amended Ethical Guidelines for Clinical Studies provided by Ministry of Health, Labor and Welfare of Japan (May 31, 2015) was approved by the *Ethics Committee*, *Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences and Okayama University Hospital.*

In situ hybridization

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

Immunohistochemistry

For immunohistochemistry, all 275 specimens were employed. Immunostaining for Spred2 was carried out using the Polink-2 plus HRP rabbit with DAB kit (GBI, Bothell, WA, USA), according to the manufacturer's instructions. In brief, sections (4-μ-thick) were treated by microwave oven in 0.1 M citric acid buffer, treated with 3%H_2O_2 in methanol, blocked with DAKO Protein Block Serum-Free (Dako, Carpinteria, CA, USA),

and incubated with anti-human Spred2 polyclonal antibody (Proteintech, Rosemont, IL, USA). Sections were then incubated with rabbit antibody specific enhancer, followed by the addition of polymer-HRP for rabbit IgG, and visualized using DAB complex. Nuclear counterstaining was performed using hematoxylin. Immunostaining for pERK1/2 (Clone D13.14.4E, Cell Signaling Technology, Danvers, MA, USA) and Ki67 (Clone MIB-1, Dako) was performed on a Ventana Discovery XT automated stainer (Ventana, Tucson, AZ, USA) with using iVIEW DAB Detection Kit (Ventana).

Evaluation of immunohistochemistry

Spred2 was stained in the cytoplasm (C) or/and membrane (M). Immunoreactivity for Spred2 was classified into 4 groups, according to subcellular localization and staining intensity; C-M-, absent or weak staining intensity in cytoplasm and membrane; C-M+, moderate to strong membranous staining without staining in cytoplasm; C+M-, moderate to strong cytoplasmic staining without membrane staining; C+M+, moderate to strong cytoplasmic and membranous staining. pERK immunostaining was scored on the following semiquantitative scale as previously reported with modifications[45]: no staining (0); focal to <10% of cells (1); 10-50% of cells (2); 50% or more cells stained weak (3); 10-50% stained strong (4); 50% or more stained strong (5). Ki67 index, a marker for cell proliferation, was determined by counting 500 tumor cells, and the percentages of positively stained cells were determined. The stained sections were assessed by two blinded pathologists.

Database analysis

Datasets with more than 25 samples in each category from Sanchez-Carbayo bladder 2[36], Blaveri bladder 2[37], and Stransky bladder[38] were used to analyze Spred2 expression in bladder cancer. Kaplan-Meier Plotter (http://www.kmplot.com) was used to analyze the prognostic values of *Spred2* mRNA expression levels in bladder carcinoma. Kaplan-Meier survival plots were drawn using data from the Kaplan-Meier database. A log-rank *p*-value <0.05 was considered to indicate a statistically significant difference.

302

303 **Statistical analysis**

304 Statistical analysis was performed using GraphPad Prism7 (GraphPad Software, San
305 Diego, CA, USA) and js-STAR (free software). Multiple comparisons were performed
306 using Dunn test with the Bonferroni correction after chi-square and Kruskal-Wallis test.
307 A residual analysis was used to identify those specific cells making the greatest
308 contribution to the chi-square test results. A value of $p<0.05$ was considered statistically
309 significant.

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Author contributions

S. Oda and A. Matsukawa planned experiments and wrote the manuscript. S. Oda, M. Fujisawa, Li. C, T. Ito, and T. Yamaguchi performed experiments and discussed the experimental findings and interpreted the results. M. Fujisawa, T. Yoshimura discussed the interpretation of the results. All authors reviewed the manuscript.

Conflict of Interest

The authors declare no competing interests.

Figure legends

Figure 1. Spred2 mRNA expression in urothelial tumors. (a) Representative photographs of HE- (original magnification 400×) and in situ hybridization-sections from each category are shown. Spred2 mRNA expression was presented by red dots. (b) The number of the red-dots per cell was counted under microscope and Spred2 mRNA expression level was shown per one cell from each category (NORMAL n=10, PUNLMP n=10, LGPUC n=15, HGPUC n=18, CIS n=18, and IUC n=14). Data were mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (Dunn test).

Figure 2. Immunohistochemical analyses of Spred2 protein expression in urothelial tumors. (a) Representative photographs of Spred2 immunohistochemistry (original magnification 400×) from each category are shown. (b) Spred2 protein expression was classified into 4 groups, according to the localization and staining intensity (C: cytoplasm, M: membrane), and %case was shown in each category (NORMAL n=101, PUNLMP n=19, LGPUC n=41, HGPUC n=43, CIS n=39, and IUC n=32). **p<0.01 (residual test). **C** Expression levels of Spred2 mRNA in each Spred2 staining pattern were shown. Data were mean ± SEM. *p<0.05, **p<0.01 (Dunn test).

Figure 3. pERK score in urothelial tumors. (a) Representative photographs of pERK immunohistochemistry (original magnification 400×) from each category are shown. (b) pERK staining intensity was evaluated and scored (0-5), and score was shown as %cases in each category (NORMAL n=101, PUNLMP n=19, LGPUC n=41, HGPUC n=43, CIS n=39, and IUC n=32). *p<0.05, **p<0.01 (residual test).

Figure 4. Ki67 index in urothelial tumors. (a) Representative photographs of Ki67 immunohistochemistry (original magnification 400×) from each category are shown. (b) Ki67 index in each category was shown (NORMAL n=101, PUNLMP n=19, LGPUC n=41, HGPUC n=43, CIS n=39, and IUC n=32). Data were mean ± SEM. *p<0.05,

461 $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ (Dunn test).

462

463 **Figure 5. Comparison between Spred2 staining and pERK score.** Spred2 and pERK
464 immunohistochemistry were carried out in each category (LGPUC n=41, HGPUC n=43,
465 CIS n=39, and IUC n=32). Spred2 staining was classified into 4 patterns (C-M-, C+M-,
466 C-M+, C+M+), according to the localization and staining intensity (C: cytoplasm, M:
467 membrane). pERK staining was scored (0-5) and %cases was shown in each Spred2
468 staining pattern. $^{**}p < 0.01$ (residual test).

469

470 **Figure 6. Comparison between Spred2 staining and Ki67 index.** Spred2 and Ki67
471 immunohistochemistry were carried out in each category (LGPUC n=41, HGPUC n=43,
472 CIS n=39, and IUC n=32). Spred2 staining was classified into 4 patterns (C-M-, C+M-,
473 C-M+, C+M+), according to the localization and staining intensity (C: cytoplasm, M:
474 membrane). Ki67 index was shown in each Spred2 staining pattern. $^{*}p<0.05$, $^{**}p<0.01$
475 (Dunn test).

476

477 **Figure 7. Spred2 expression in overall survival of patients with bladder cancer.** (a)
478 Statistical analyses of Spred2 expression in normal, superficial bladder cancer
479 (superficial) and infiltrating bladder urothelial carcinoma (infiltrating) from 3 different
480 datasets (Sanchez-Carbayo bladder 2, Blaveri bladder 2, and Stransky bladder) were
481 shown. The numbers in parentheses indicates the number of samples. $^{**}p<0.01$,
482 $^{****}p<0.0001$, unpaired two-tailed t test. (b,c) Kaplan-Meier analysis of the data in
483 www.kmplot.com was used to determine the survival probability for 30 months (b) and
484 150 months (c) of patients with high or low Spred2 expression, followed by the log-rank
485 test.

Table 1. Cases for the enrolled 275 patients.

	cases	number of examined	features		
	number (%)	by IHC/ISH	progression	nuclear grade	invasiveness
NORMAL	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

NORMAL; normal 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; invasive urothelial carcinoma. IHC; immunohistochemistry, ISH; in situ hybridization.

Table 2. Subcellular immunolocalization of Spred2

	cytoplasm	membrane	Case (%)
C-M-	-	-	88 (32.0)
C+M-	+	-	122 (44.4)
C-M+	-	+	54 (19.6)
C+M+	+	+	11 (4.0)

Fig. 1

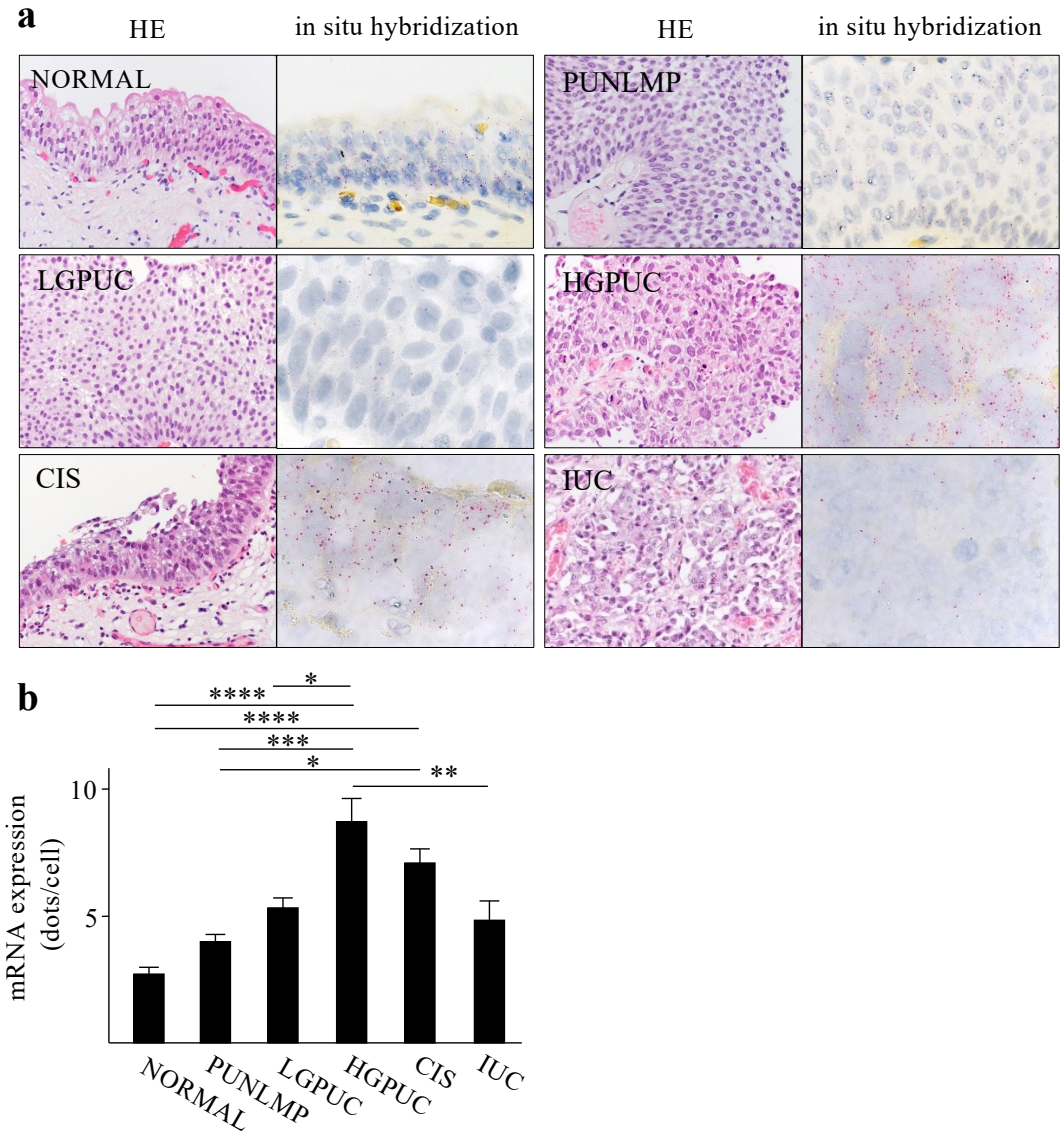

a
HE
in situ hybridization
HE
in situ hybridization
NORMAL
PUNLMP
LGPUC
HGPUC
CIS
IUC
b
****
*
****
***
*
**
mRNA expression
(dots/cell)
10
5
NORMAL
PUNLMP
LGPUC
HGPUC
CIS
IUC

Fig. 2

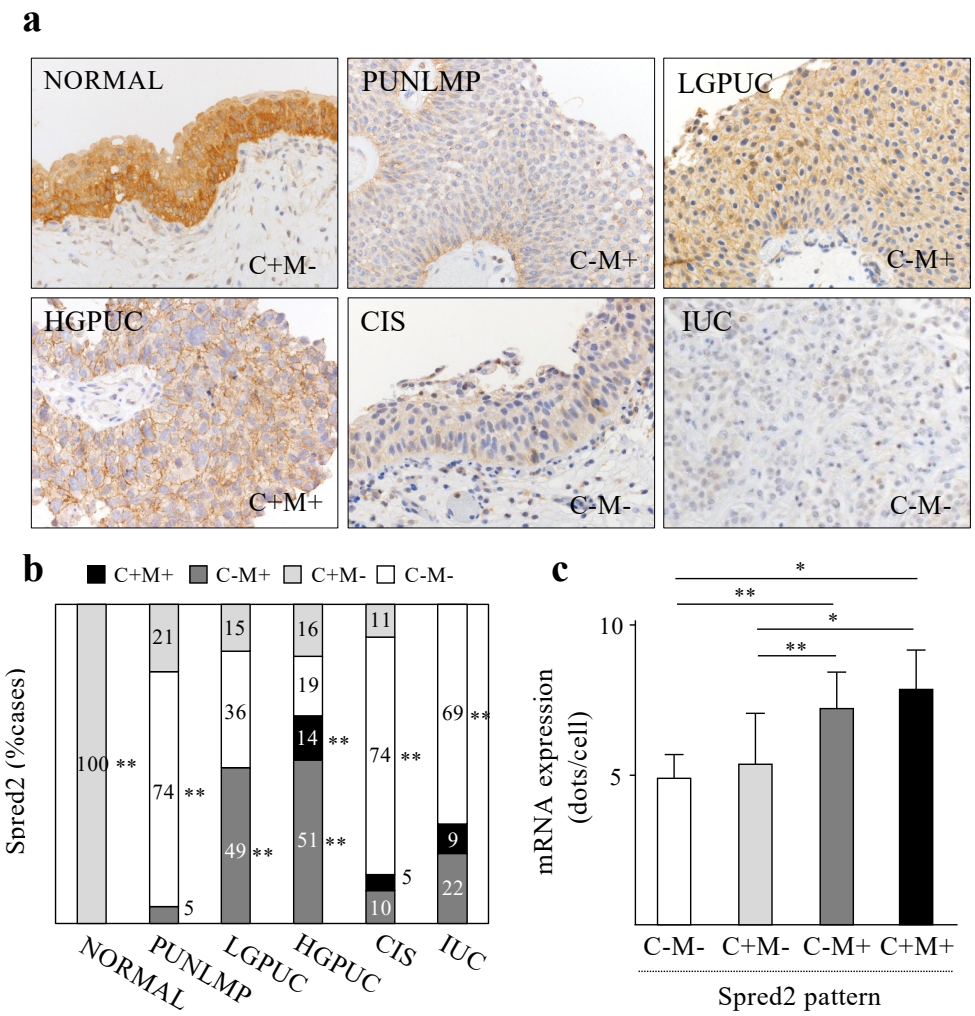

a
NORMAL
C+M-
PUNLMP
C-M+
LGPUC
C-M+
HGPUC
C+M+
CIS
C-M-
IUC
C-M-
b
C+M+
C-M+
C+M-
C-M-
Spred2 (%cases)
100 **
21
74 **
5
15
36
49 **
16
19
14 **
51 **
11
74 **
5
10
69 **
9
22
NORMAL
PUNLMP
LGPUC
HGPUC
CIS
IUC
c
*
**
*
**
10
5
mRNA expression
(dots/cell)
C-M-
C+M-
C-M+
C+M+
Spred2 pattern

Fig. 3

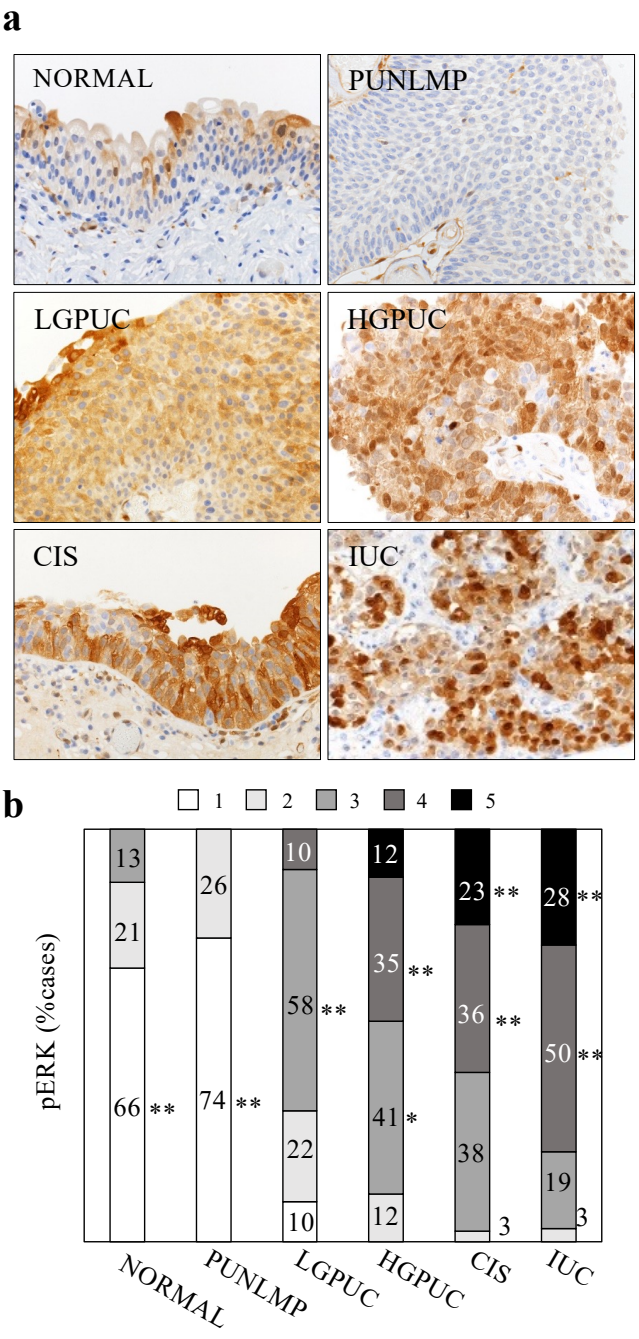

a
NORMAL
PUNLMP
LGPUC
HGPUC
CIS
IUC
b
1
2
3
4
5
pERK (%cases)
13
21
66 **
26
74 **
10
58 **
22
10
12
35 **
41 *
12
23 **
36 **
38
3
28 **
50 **
19
3
NORMAL
PUNLMP
LGPUC
HGPUC
CIS
IUC

Fig. 4

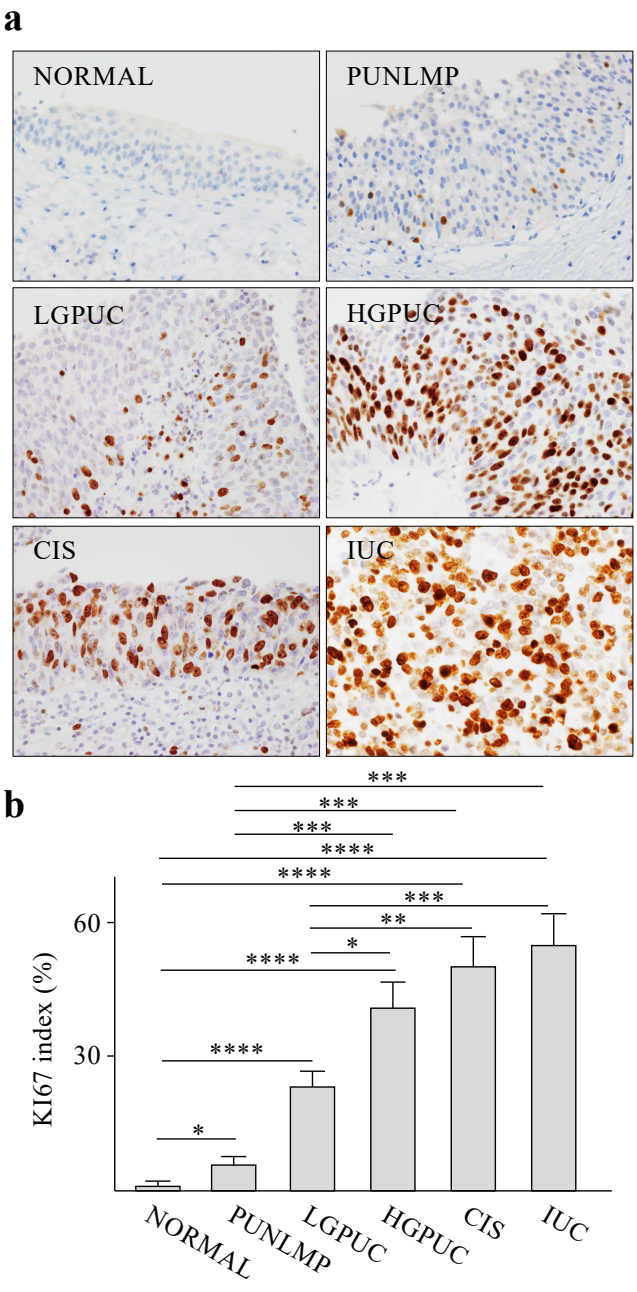

a
NORMAL
PUNLMP
LGPUC
HGPUC
CIS
IUC
b
KI67 index (%)
60
30
***
***
***
****
****
***
**
*
****
****
*
NORMAL
PUNLMP
LGPUC
HGPUC
CIS
IUC

Fig. 5

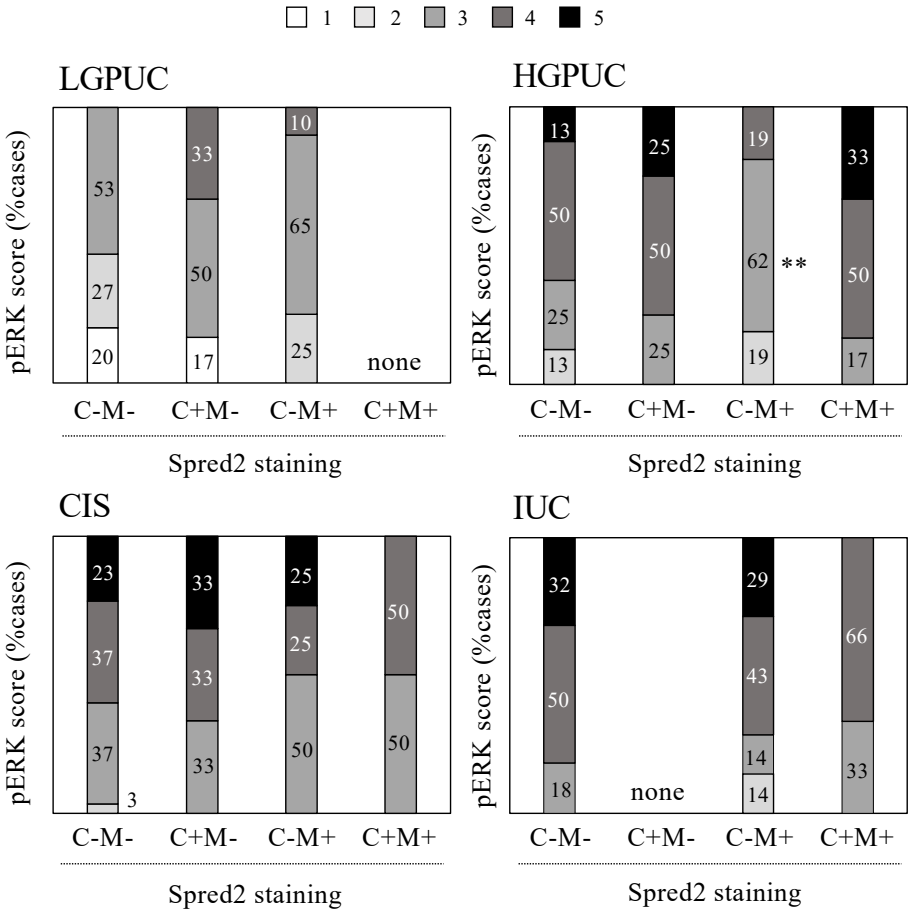

1
2
3
4
5
LGPUC
HGPUC
CIS
IUC
pERK score (%cases)
C-M-
C+M-
C-M+
C+M+
Spred2 staining
53
27
20
33
50
17
10
65
25
none
13
50
25
13
25
50
25
19
62
19
**
33
50
17
23
37
37
3
33
33
33
25
25
50
50
50
32
50
18
none
29
43
14
14
66
33

Fig. 6

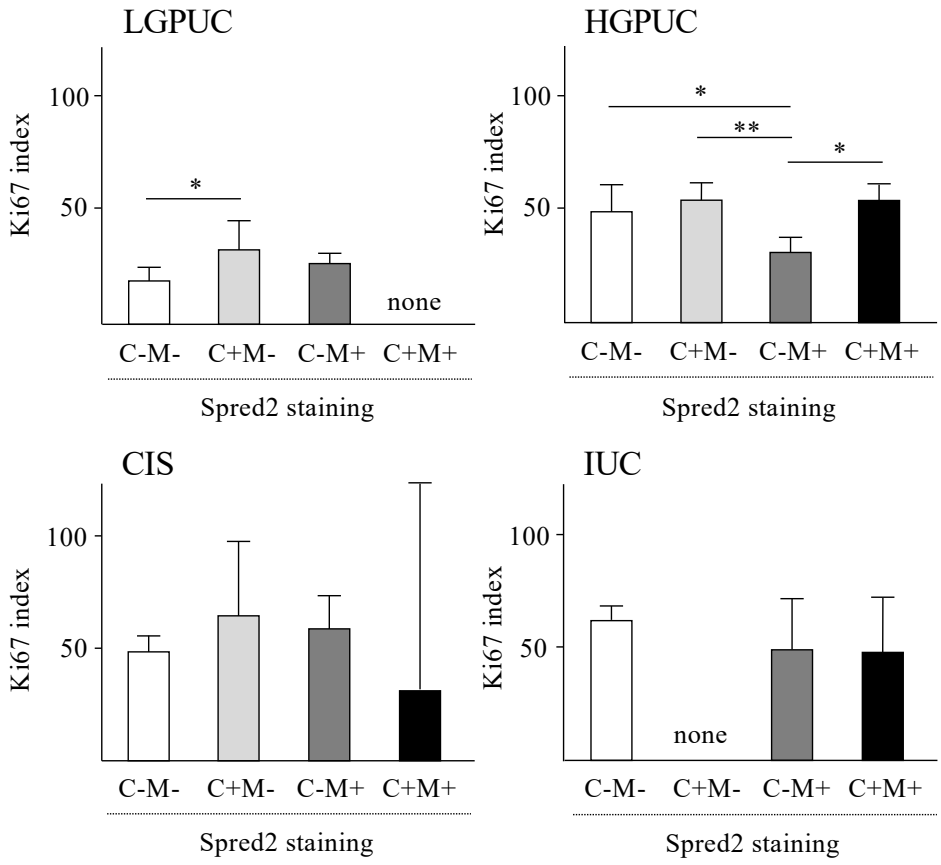
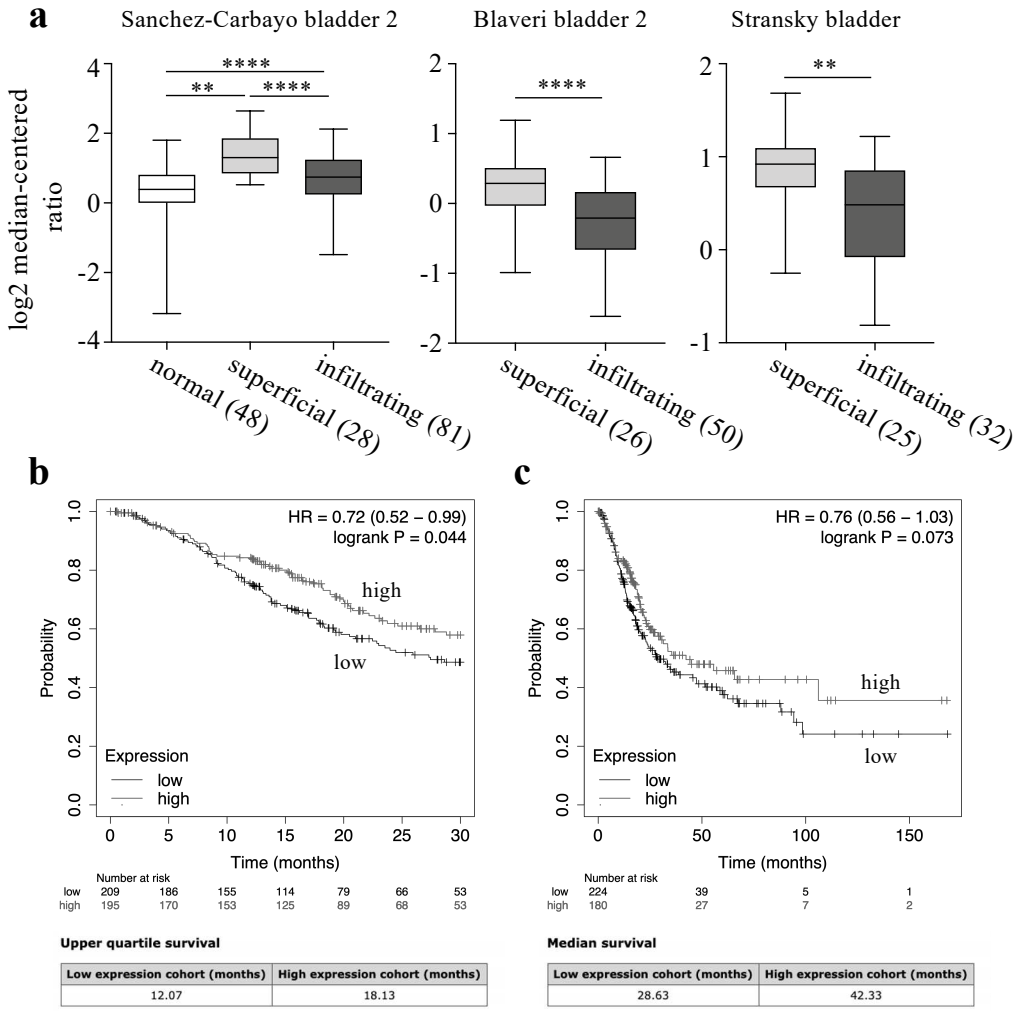

LGPUC
HGPUC
CIS
IUC
Ki67 index
100
50
*
**
none
C-M-
C+M-
C-M+
C+M+
Spred2 staining

Fig. 7



Upper quartile survival

Low expression cohort (months)	High expression cohort (months)
12.07	18.13

Median survival

Low expression cohort (months)	High expression cohort (months)
28.63	42.33

Figures

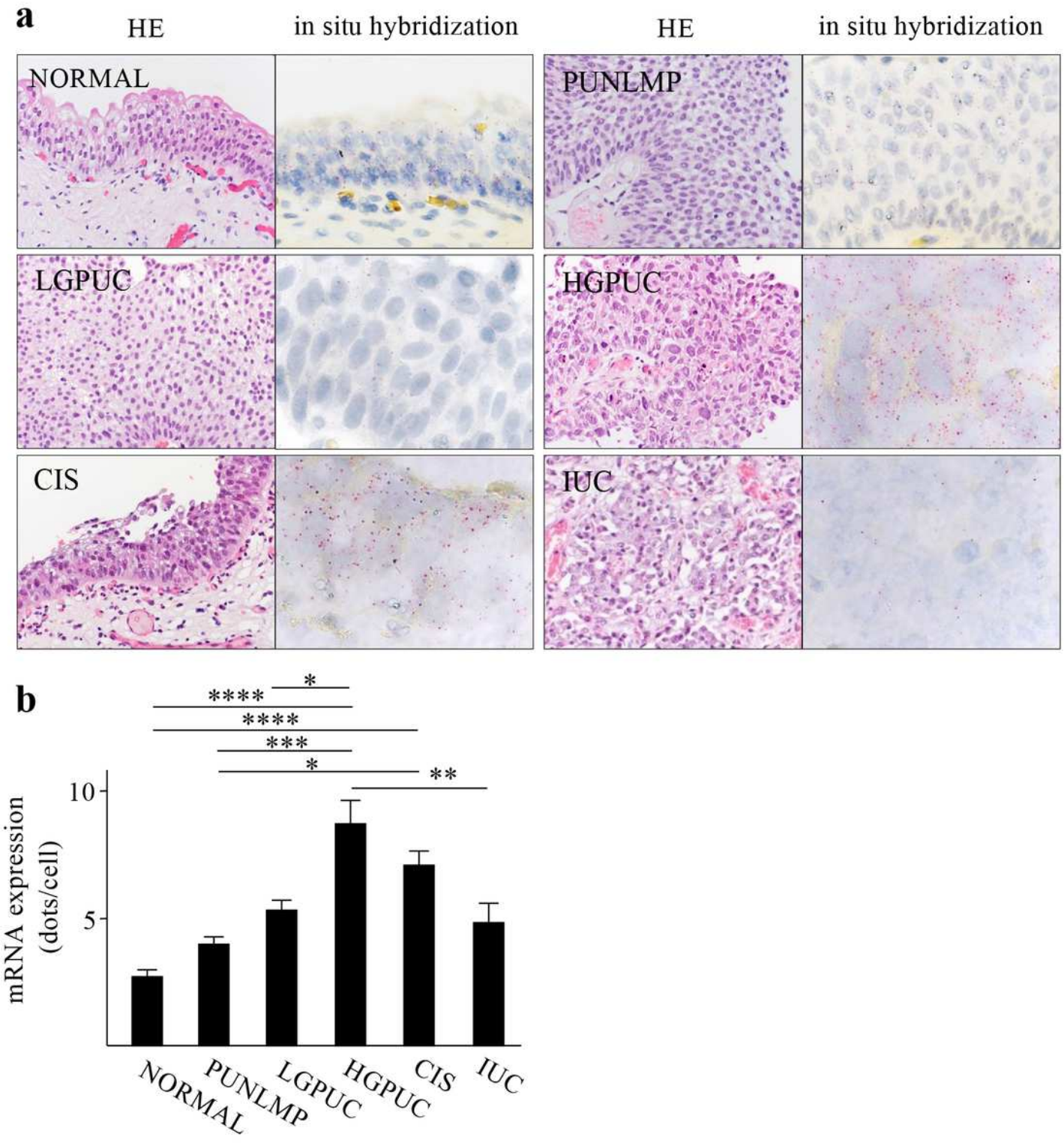


Figure 1

Spred2 mRNA expression in urothelial tumors. (a) Representative photographs of HE- (original magnification 400×) and in situ hybridization-sections from each category are shown. Spred2 mRNA expression was presented by red dots. (b) The number of the red-dots per cell was counted under

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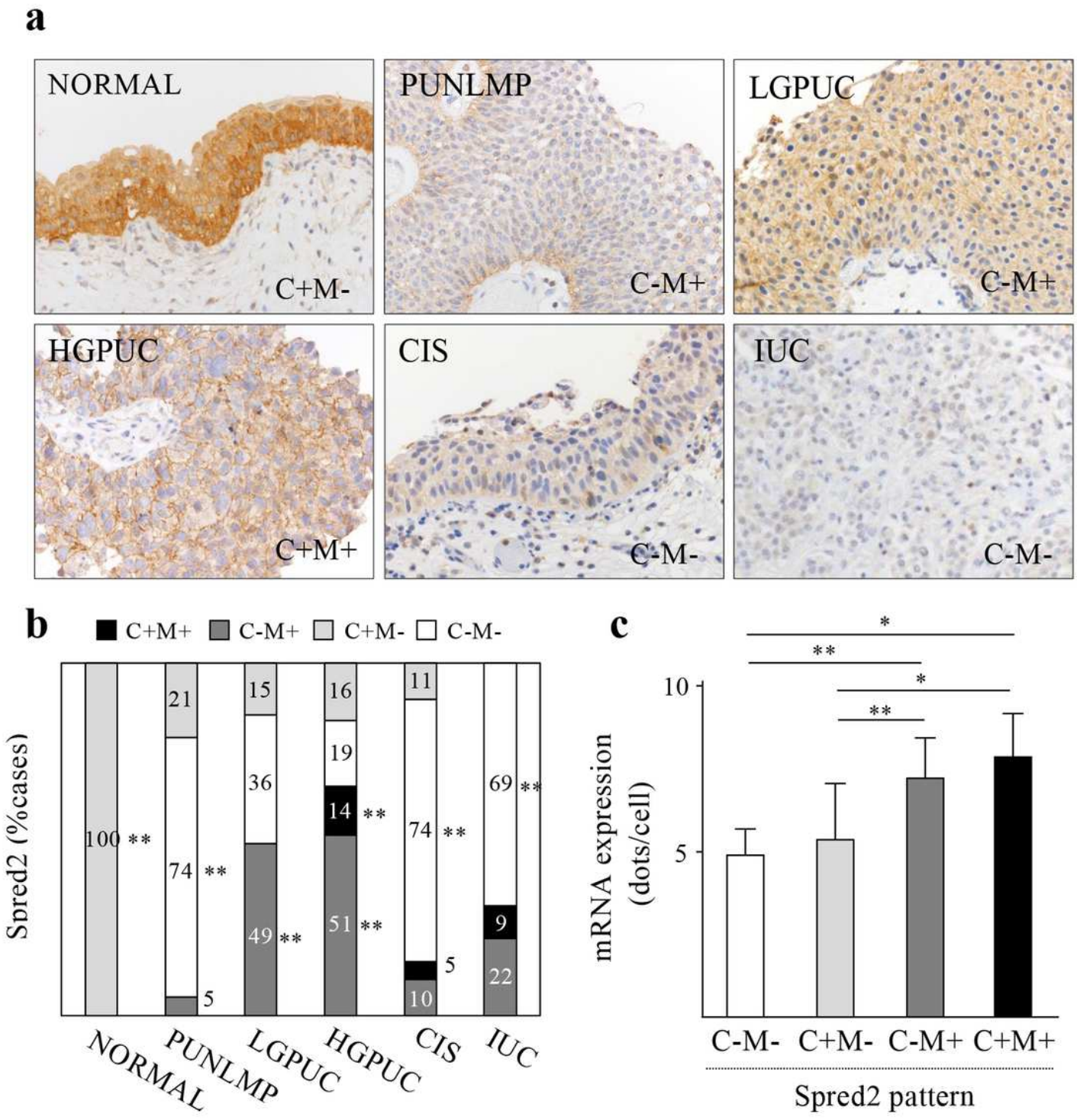

Figure 2

Immunohistochemical analyses of Spred2 protein expression in urothelial tumors. (a) Representative photographs of Spred2 immunohistochemistry (original magnification 400×) from each category are

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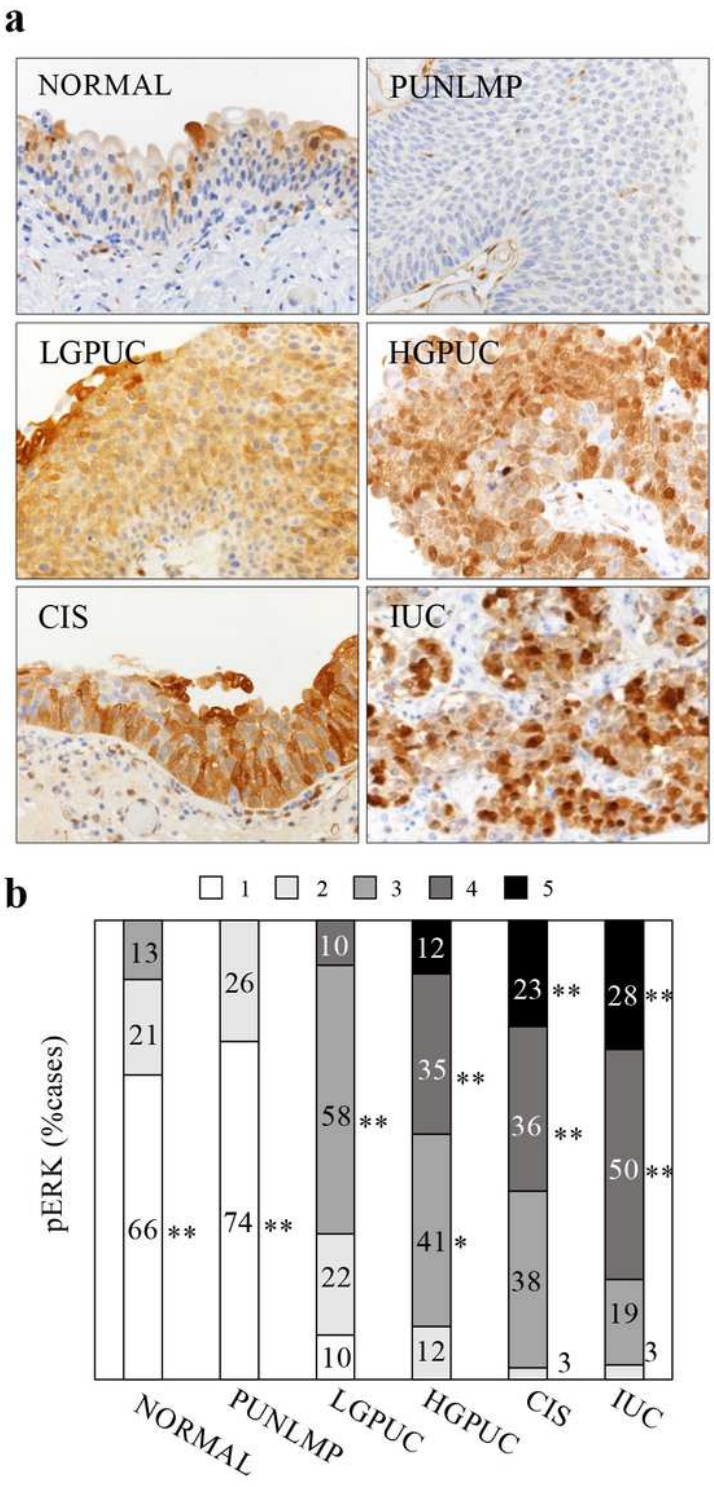

Figure 3

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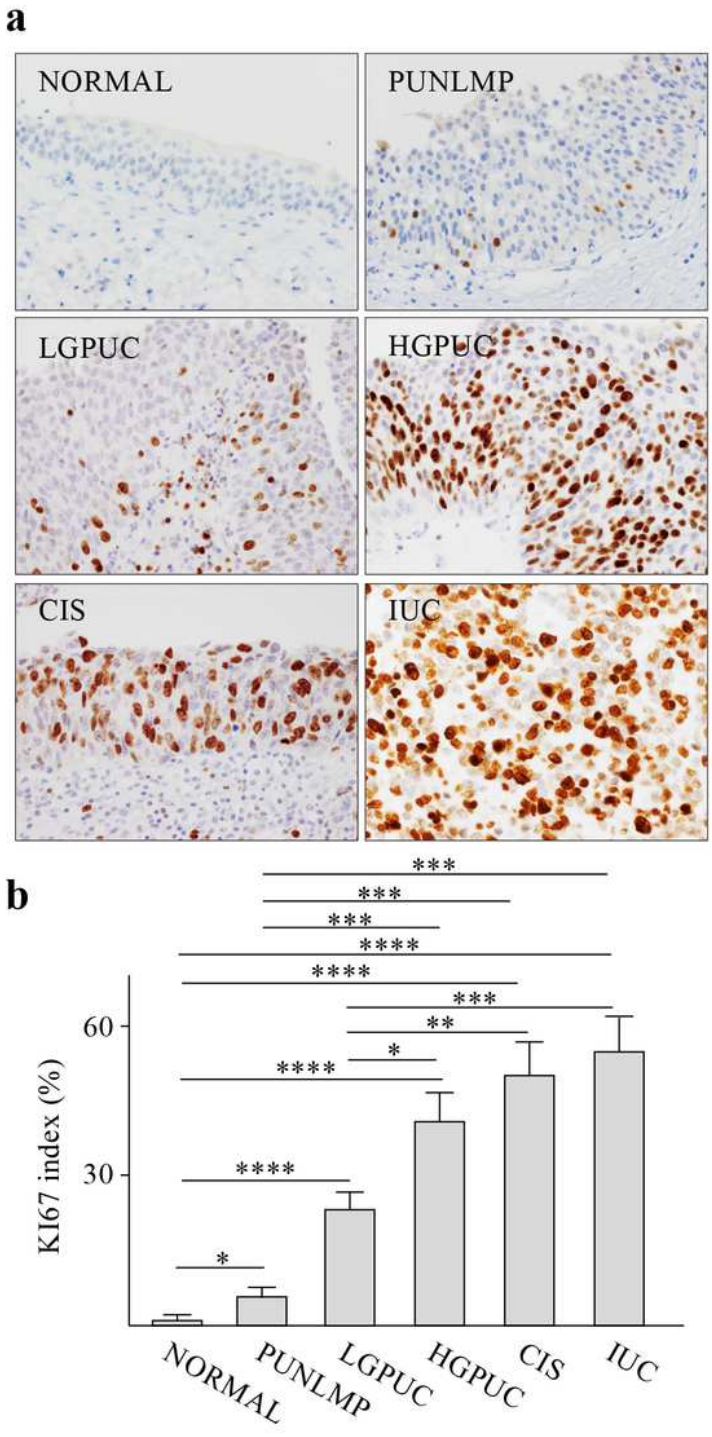

Figure 4

Ki67 index in urothelial tumors. (a) Representative photographs of Ki67 immunohistochemistry (original magnification 400×) from each category are shown. (b) Ki67 index in each category was shown (NORMAL n=101, PUNLMP n=19, LGPUC n=41, HGPUC n=43, CIS n=39, and IUC n=32). Data were mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (Dunn test).

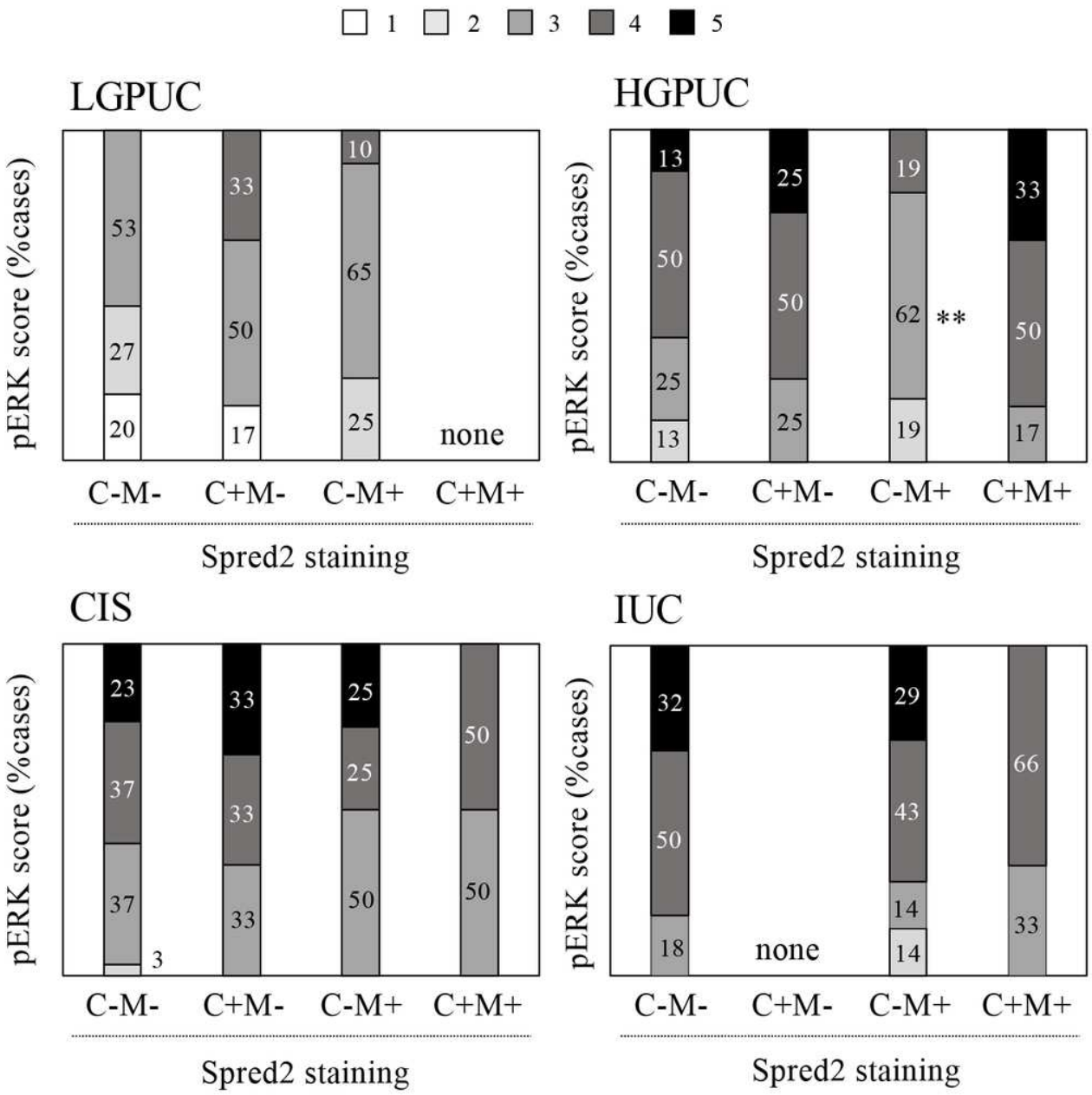

Figure 5

Comparison between Spred2 staining and pERK score. Spred2 and pERK immunohistochemistry were carried out in each category (LGPUC n=41, HGPUC n=43, CIS n=39, and IUC n=32). Spred2 staining was

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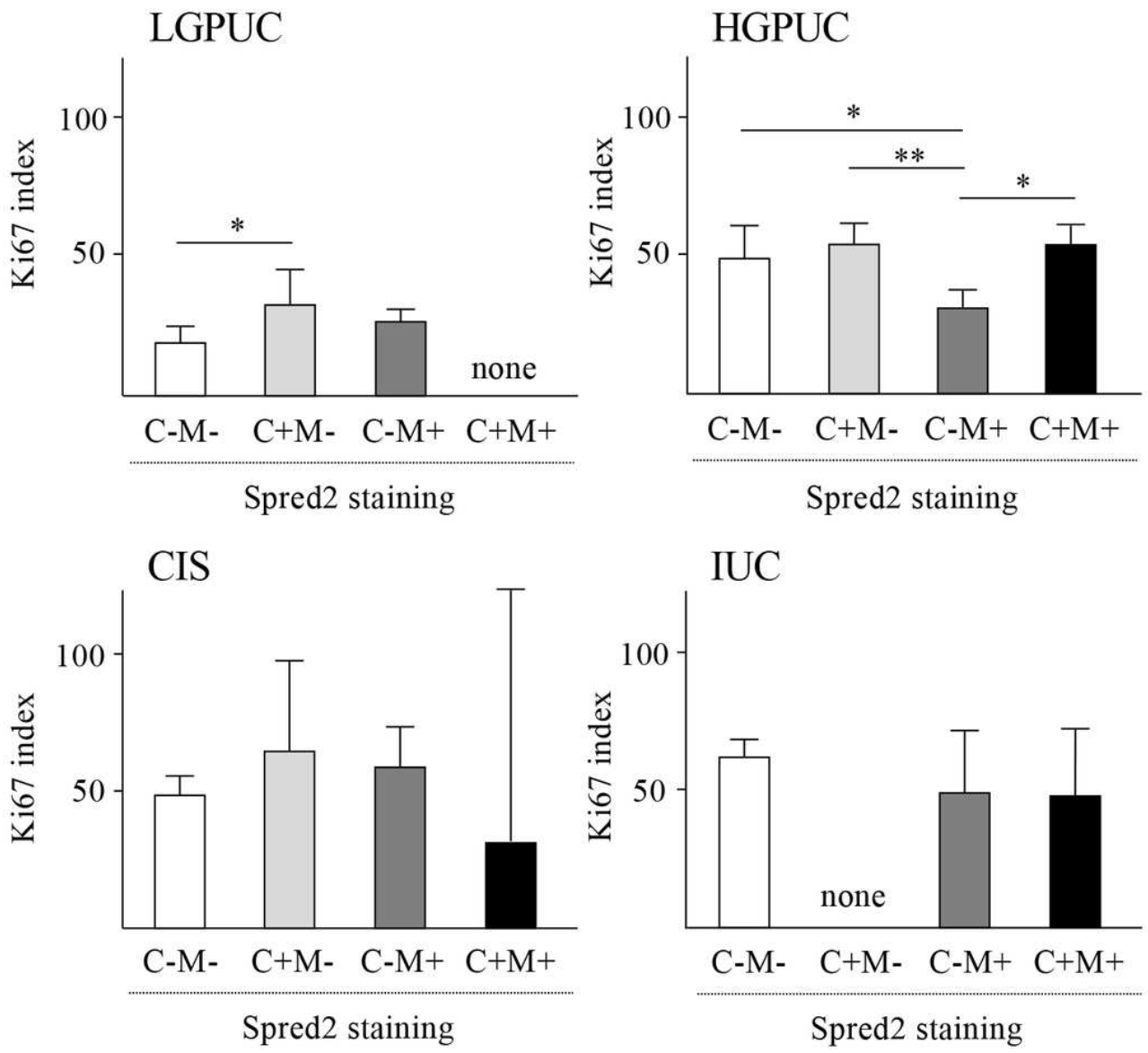

Figure 6

Comparison between Spred2 staining and Ki67 index. Spred2 and Ki67 immunohistochemistry were carried out in each category (LGPUC n=41, HGPUC n=43, CIS n=39, and IUC n=32). Spred2 staining was classified into 4 patterns (C-M-, C+M-, C-M+, C+M+), according to the localization and staining intensity (C: cytoplasm, M: membrane). Ki67 index was shown in each Spred2 staining pattern. *p<0.05, **p<0.01 (Dunn test).

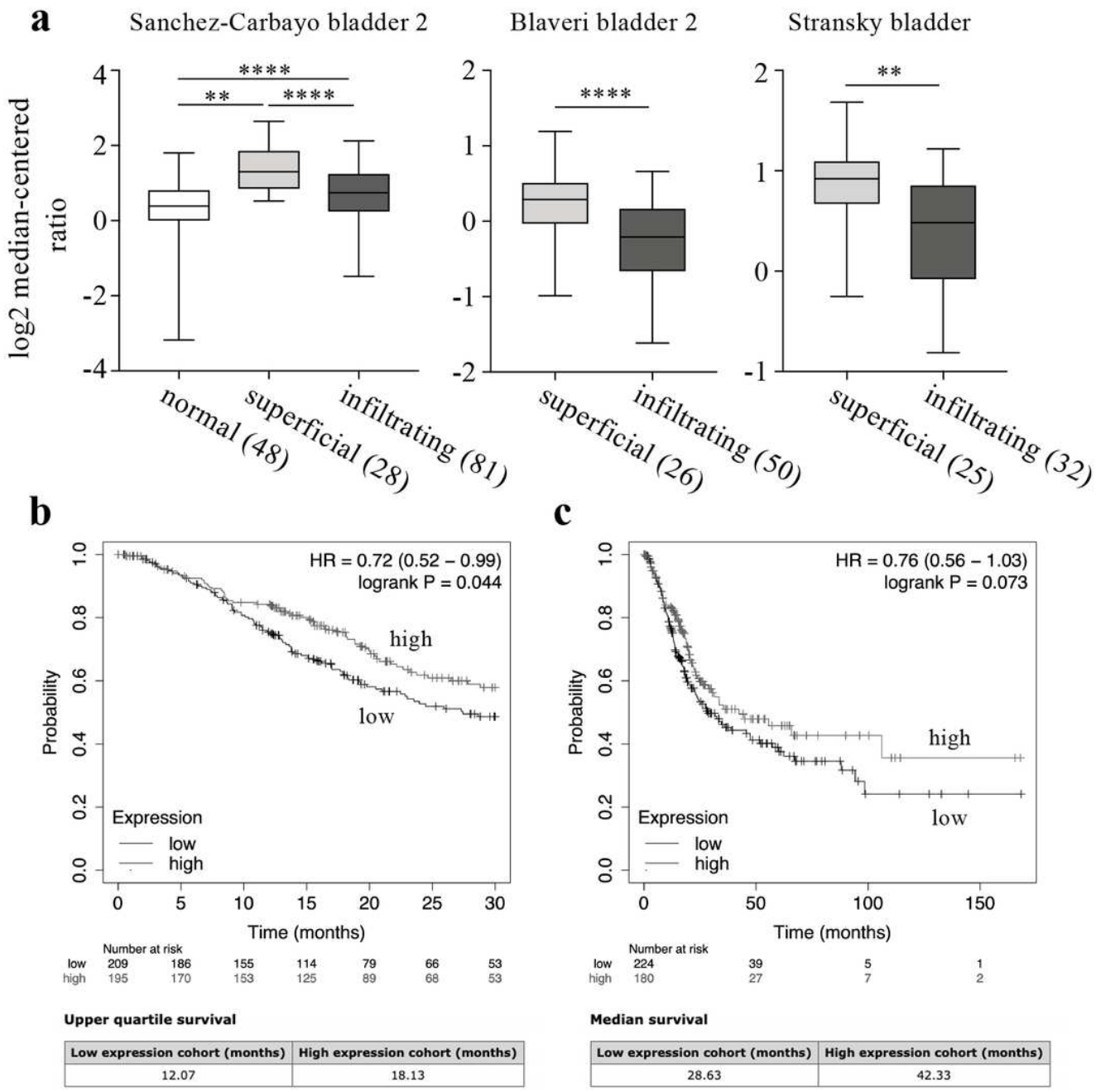

Figure 7

Spred2 expression in overall survival of patients with bladder cancer. (a) Statistical analyses of Spred2 expression in normal, superficial bladder cancer (superficial) and infiltrating bladder urothelial carcinoma (infiltrating) from 3 different datasets (Sanchez-Carbayo bladder 2, Blaveri bladder 2, and Stransky bladder) were shown. The numbers in parentheses indicates the number of samples. **p<0.01, ****p<0.0001, unpaired two-tailed t test. (b,c) Kaplan-Meier analysis of the data in www.kmplot.com was used to determine the survival probability for 30 months (b) and 150 months (c) of patients with high or low Spred2 expression, followed by the log-rank test.

Supplementary Files

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- SupplementaryFigures.pdf