Integrated analysis of tertiary lymphoid structures in relation to tumor-infiltrating lymphocytes and patient survival in pancreatic ductal adenocarcinoma

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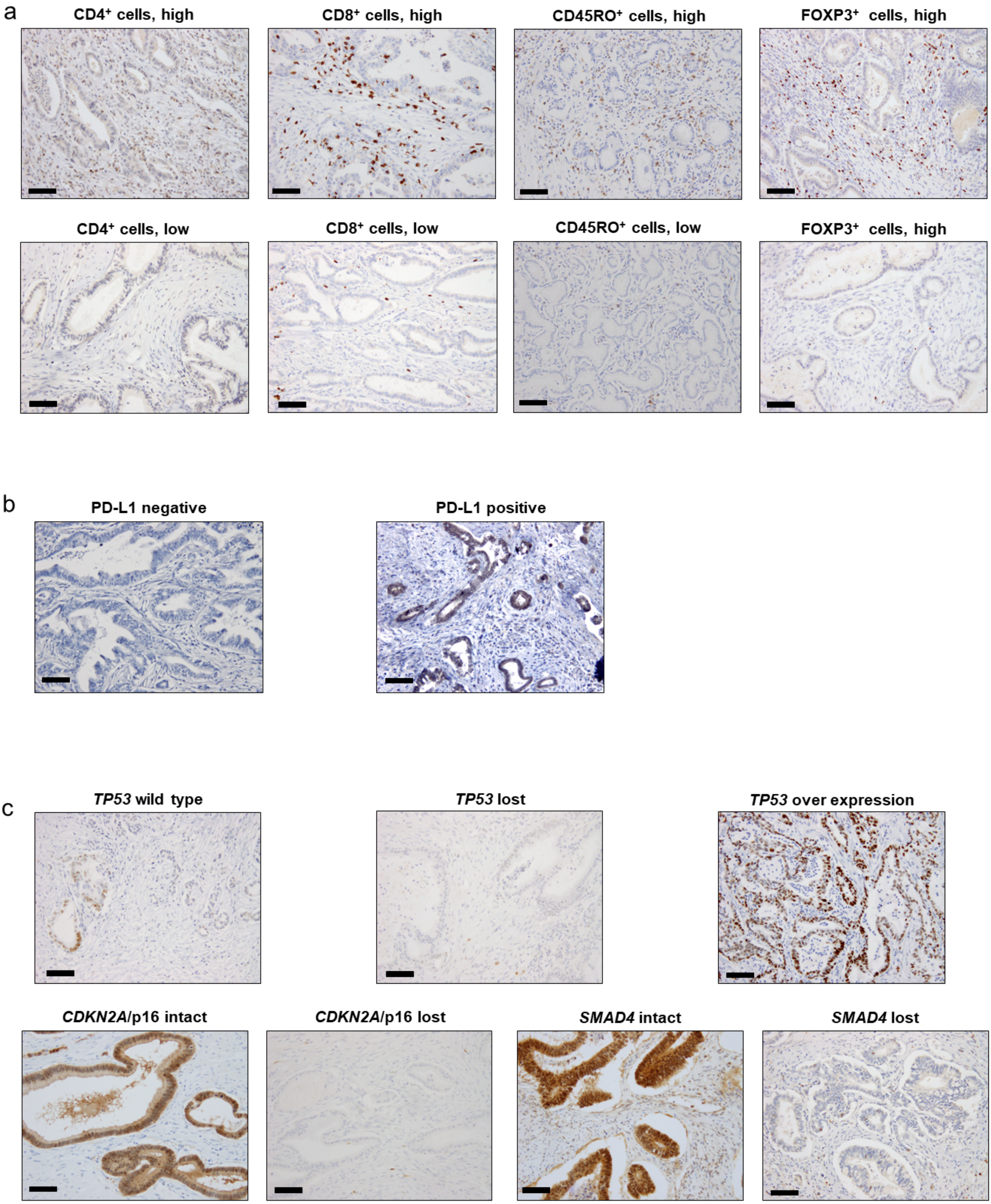
Supplementary Fig. 1



CD4$^+$ cells, high

CD8$^+$ cells, high

CD45RO$^+$ cells, high

FOXP3$^+$ cells, high

CD4$^+$ cells, low

CD8$^+$ cells, low

CD45RO$^+$ cells, low

FOXP3$^+$ cells, high

PD-L1 negative

PD-L1 positive

TP53 wild type

TP53 lost

TP53 over expression

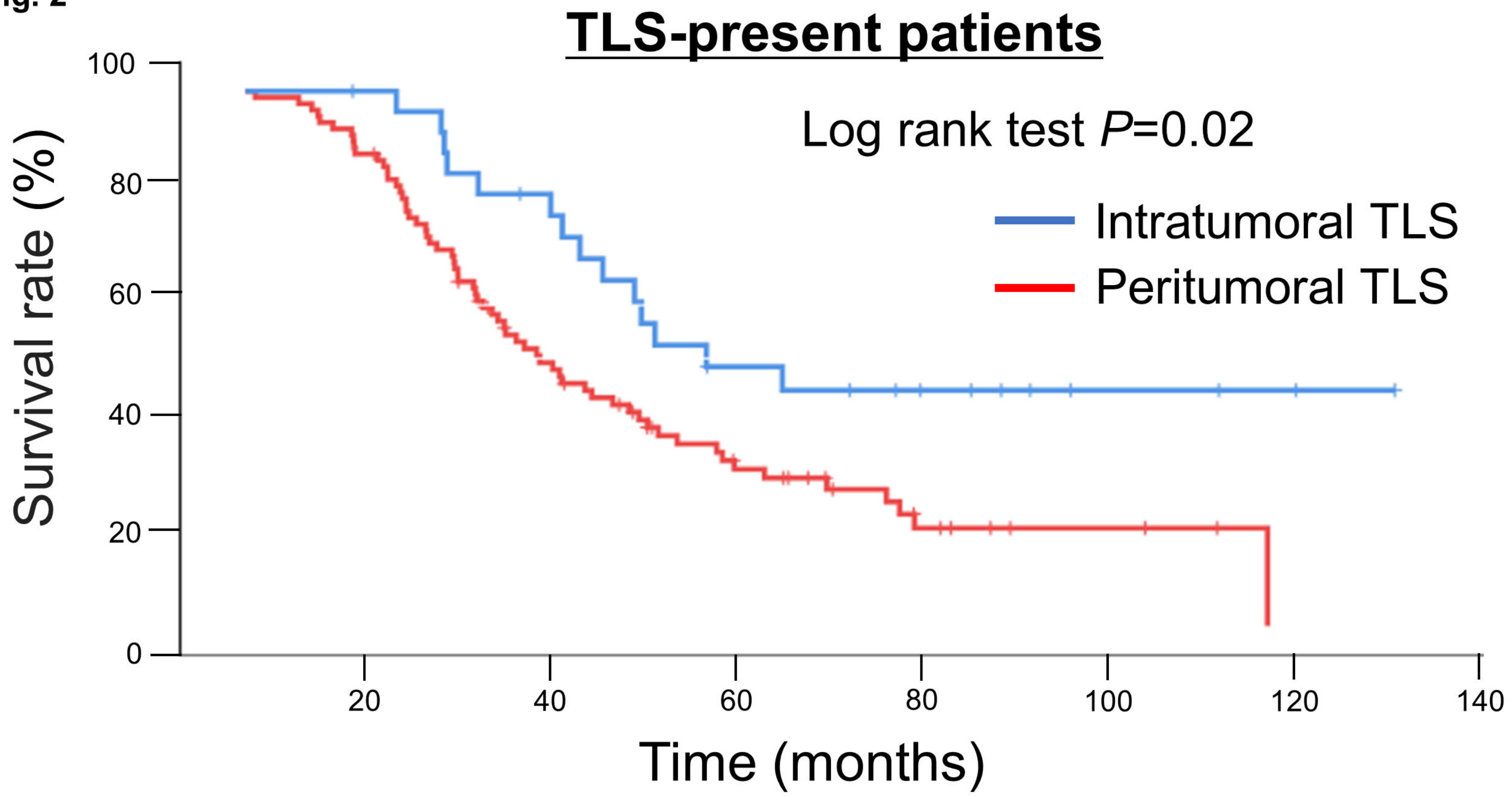
CDKN2A/p16 intact

CDKN2A/p16 lost

SMAD4 intact

SMAD4 lost

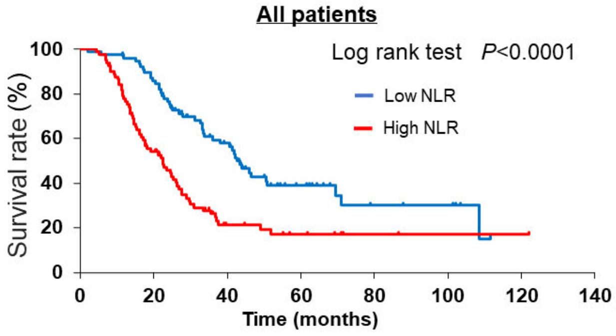
Supplementary Fig. 2



Time (months)	0	20	40	60	80	100	120
Intratumoral TLS	27	25	16	10	6	3	1
Peritumoral TLS	85	60	30	14	4	2	0

Supplementary Fig. 3

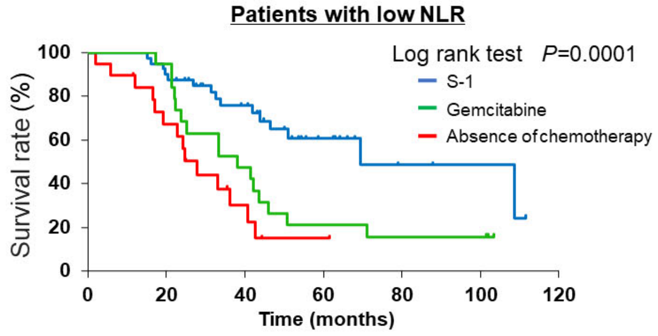
a



Number of samples

Time (months)	0	20	40	60	80	100	120
Low NLR	80	66	37	15	6	5	0
High NLR	82	42	11	6	2	1	1

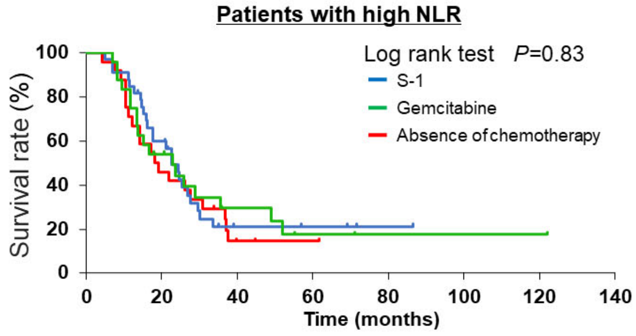
b



Number of samples

Time (months)	0	20	40	60	80	100	120
S-1	41	36	24	10	3	2	0
Gemcitabin	19	18	9	4	3	3	0
No chemotherapy	20	12	4	1	0	0	0

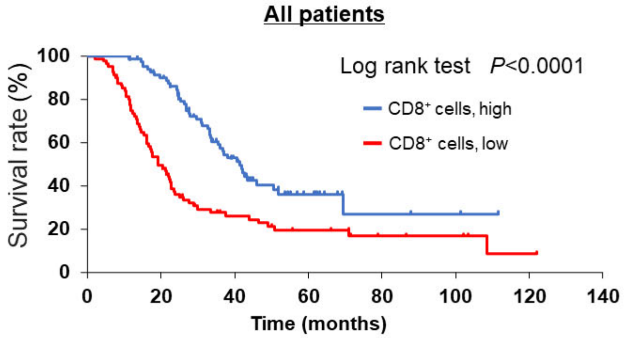
c



Number of samples

Time (months)	0	20	40	60	80	100	120
S-1	33	19	3	2	1	0	0
Gemcitabin	24	12	5	2	1	1	1
No chemotherapy	25	11	2	1	0	0	0

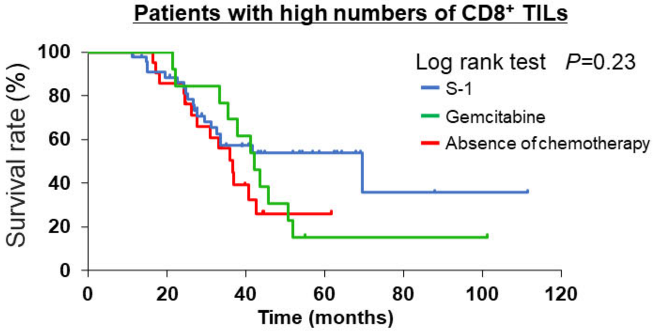
d



Number of samples

Time (months)	0	20	40	60	80	100	120
$CD8^+$ cells, high	81	69	32	11	3	2	0
$CD8^+$ cells, low	81	39	16	10	5	4	1

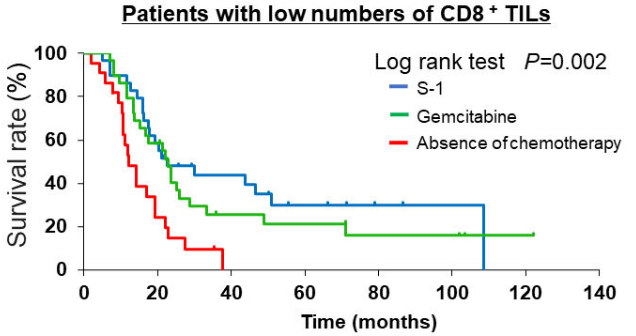
e



Number of samples

Time (months)	0	20	40	60	80	100	120
S-1	45	38	18	8	2	1	0
Gemcitabin	14	8	2	1	1	1	0
No Chemotherapy	22	18	6	2	0	0	0

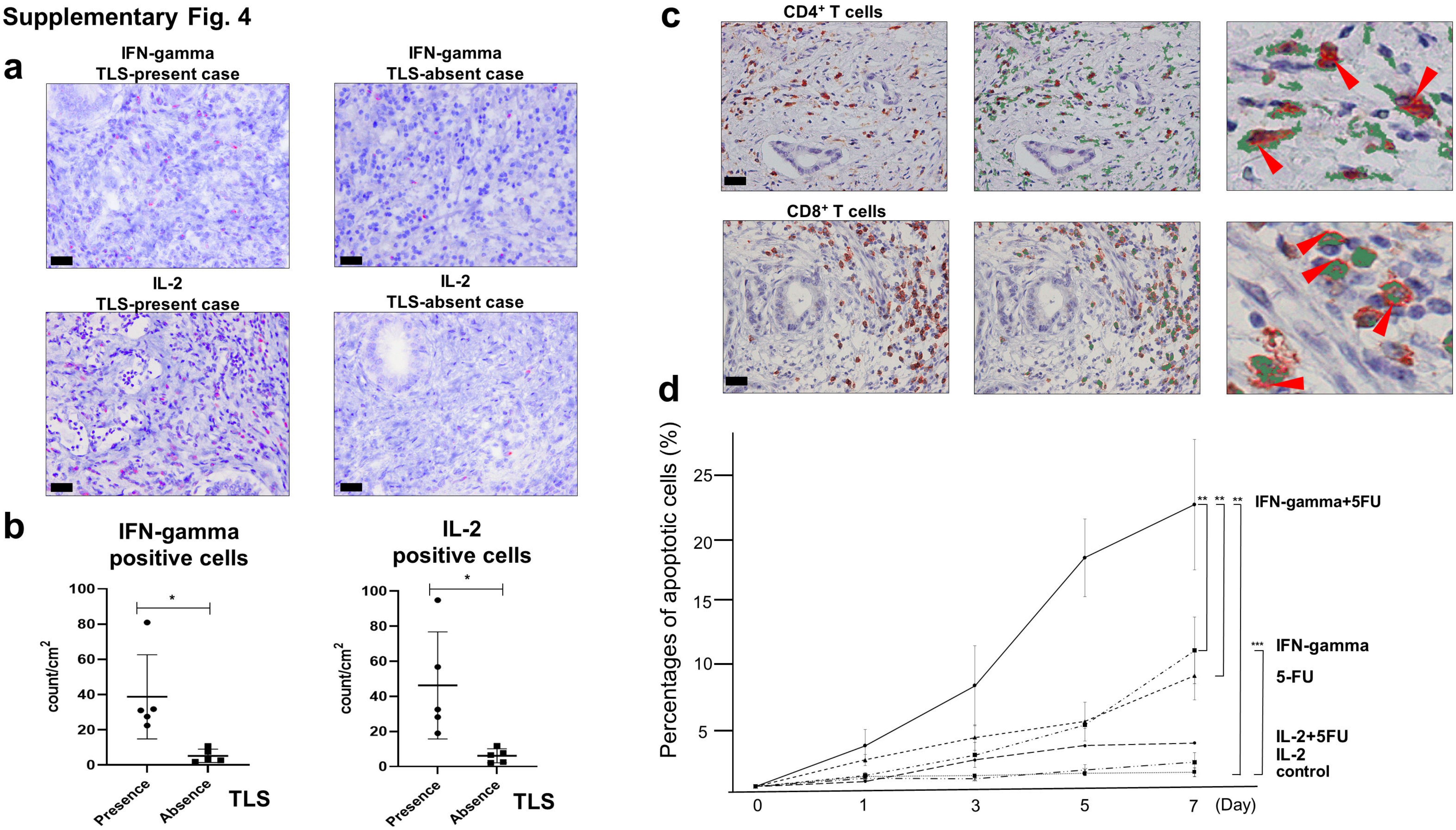
f



Number of samples

Time (months)	0	20	40	60	80	100	120
S-1	29	17	10	5	2	1	0
Gemcitabin	29	17	6	5	3	3	1
No Chemotherapy	23	5	0	0	0	0	0

Supplementary Fig. 4



Supplementary Table 1. The frequency of *KRAS* mutations by next-generation sequencing, and the association between *KRAS* mutations and TLS

***KRAS* mutation**	**No. patients (%)**	TLS		***P* value**
		Present N=104 (%)	**Absent** N=46, (%)	
G12D	56 (37.2%)	33 (31.7%)	23 (50.0%)	0.044
G12V	51 (34.0%)	37 (35.6%)	14 (30.4%)	0.85
G12R	28 (18.7%)	23 (22.1%)	5 (10.9%)	0.12
G12C	3 (2.0%)	3 (2.9%)	0 (0.0%)	0.55
Q61H	9 (6.0%)	6 (5.8%)	3 (6.5%)	0.99
Others	3 (2.0%)	2 (1.9%)	1 (2.2%)	0.99

Others include Q61L, Q61R and Q61K.

Supplementary Table 2. Integrated Classification of *TP53*, *CDKN2A* and *SMAD4* using next-generation sequencing, Droplet Digital PCR and Immunohistochemistry

Gene			Molecular wild type	Molecular altered[b]
TP53[a]	IHC	Wild type	44	0
		Overexpression	7	80
		Loss	16	15
CDKN2A/p16[a]	IHC	Wild type	53	5
		Loss	29	75
SMAD4[a]	IHC	Wild type	88	12
		Loss	10	52

[a] For *TP53*, *CDKN2A* and *SMAD4,* the final classification of gene alterations was analyzed by comprehensively considering the NGS data, ddPCR data and the IHC expression pattern.

[b] Molecularly altered includes SNV or short indels or CNV.

Gray highlight indicates cases with presence of final integrated gene alterations. ddPCR, droplet digital PCR; IHC, immunohistochemistry; NGS, next-generation sequencing.

Supplementary Table 3. The association of TLS with blood biochemical parameters

Blood biochemical parameters	**All patients** (N=162)	**TLS**		***P* value**
		Present (N=112)	**Absent** (N=50)	
CRP				0.16
< 0.14 (mg/dl)	102 (63.0%)	75 (67.0%)	27 (54.0%)	
≥ 0.14 (mg/dl)	60 (37.0%)	37 (33.0%)	23 (46.0%)	
TP				0.61
< 6.7 (g/dl)	73 (45.1%)	52 (46.4%)	21 (42.0%)	
≥ 6.7 (g/dl)	89 (54.9%)	60 (53.6%)	29 (58.0%)	
Albumin				0.35
< 4.1 (g/dl)	48 (29.6%)	36 (32.1%)	12 (24.0%)	
≥ 4.1 (g/dl)	114 (70.4%)	76 (67.9%)	38 (76.0%)	
AST				0.11
< 31 (IU/L)	124 (76.5%)	90 (80.4%)	34 (68.0%)	
≥ 31 (IU/L)	38 (23.5%)	22 (19.6%)	16 (32.0%)	
ALT				0.10
M:< 23 (IU/L), F< 43 (IU/L)	109 (67.3%)	80 (71.4%)	29 (58.0%)	
M:≥ 23 (IU/L), F≥ 43 (IU/L)	53 (32.7%)	32 (28.6%)	21 (42.0%)	
Total Bilirubin				0.17
< 1.5 (mg/dl)	136 (84.0%)	97 (86.6%)	39 (78.0%)	
≥ 1.5 (mg/dl)	26 (16.0%)	15 (13.4%)	11 (22.0%)	
BUN				0.28
< 20 (mg/dl)	144 (88.9%)	97 (86.6%)	47 (94.0%)	
≥ 20 (mg/dl)	18 (11.1%)	15 (13.4%)	3 (6.0%)	
Creatinine				0.25
M:< 0.8 (IU/L), F< 1.08 (IU/L)	119 (73.5%)	79 (70.5%)	40 (80.0%)	
M:≥ 0.8 (IU/L), F≥ 1.08 (IU/L)	43 (26.5%)	33 (29.5%)	10 (20.0%)	

(%) indicates the proportion of cases with blood biochemical parameters according to the presence or absence of TLS.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CRP, C-reactive protein; F, female; M, male.

Supplementary Table 4. Presence of TLS and overall mortality in PDAC

			Overall mortality		
	No. of cases	No. of events	Univariate HR (95%CI)	Multivariate HR[a] (95%CI)	Multivariate HR[b] (95%CI)
All cases	162	117			
TLS absence	50	44	1 (reference)	1 (reference)	1 (reference)
TLS presence	112	73	0.44 (0.30-0.65)	0.45 (0.30-0.68)	0.42 (0.28-0.62)
P value			<0.0001	0.0002	<0.0001
Pathological stage IIb-III[c]	119	94			
TLS absence	40	38	1 (reference)	1 (reference)	1 (reference)
TLS presence	79	56	0.36(0.24-0.56)	0.36 (0.23-0.57)	0.35 (0.23-0.54)
P value			<0.0001	<0.0001	<0.0001

[a] The hazard ratio was initially adjusted for age, sex, BMI, amount of alcohol consumption, family history of pancreatic cancer, history of smoking, diabetes mellitus, serum CA19-9, serum CEA.

[b] The hazard ratio was initially adjusted for stage, histology, adjuvant, neo-adjuvant, residual tumor status, gene alterations (*KRAS*, *TP53*, *CDKN2A*, *SMAD4*).

[c] Pathological stage was diagnosed based on TNM Classification of Malignant Tumors, 8th Edition. HR, hazard ratio; PDAC, pancreatic ductal adenocarcinoma; TLS, tertiary lymphoid structure.

Supplementary Table 5. Local immune markers (the levels of CD4$^+$, CD8$^+$, CD45RO$^+$ and FOXP$^+$ TILs) or systemic immune markers (serum ALC, ANC and NLR) and patient mortality in PDAC

		No. of cases	No. of events	Pancreatic cancer specific mortality			No. of events	Overall mortality		
				Univariate HR (95%CI)	Multivariate HR^a (95%CI)	Multivariate HR^b (95%CI)		Univariate HR (95%CI)	Multivariate HR^a (95%CI)	Multivariate HR^b (95%CI)
CD4		162	107				117			
	Low	82	65	1 (reference)	1 (reference)	1 (reference)	70	1 (reference)	1 (reference)	1 (reference)
	High	80	42	0.38 (0.25-0.56)	0.32 (0.21-0.48)	0.40 (0.25-0.62)	47	0.39 (0.26-0.56)	0.33 (0.22-0.49)	0.41 (0.26-0.63)
	P value			<0.0001	<0.0001	<0.0001		<0.0001	<0.0001	<0.0001
CD8		162	107				117			
	Low	81	63	1 (reference)	1 (reference)	1 (reference)	66	1 (reference)	1 (reference)	1 (reference)
	High	81	44	0.42 (0.29-0.62)	0.41 (0.27-0.60)	0.31 (0.20-0.47)	51	0.48 (0.33-0.69)	0.47 (0.32-0.68)	0.35 (0.24-0.53)
	P value			<0.0001	<0.0001	<0.0001		<0.0001	<0.0001	<0.0001
CD45RO		162	107							
	Low	81	66	1 (reference)	1 (reference)	1 (reference)		1 (reference)	1 (reference)	1 (reference)
	High	81	41	0.41 (0.28-0.61)	0.41 (0.27-0.62)	0.50 (0.33-0.76)		0.48 (0.33-0.69)	0.48 (0.32-0.71)	0.57 (0.38-0.85)
	P value			<0.0001	<0.0001	0.001		<0.0001	0.0002	0.006
FOXP3		162	107				117			
	Low	81	60	1 (reference)	1 (reference)	1 (reference)	64	1 (reference)	1 (reference)	1 (reference)
	High	81	47	0.76 (0.52-1.12)	0.95 (0.62-1.46)	0.88 (0.59-1.31)	53	0.80 (0.55-1.16)	0.92 (0.61-1.39)	0.90 (0.61-1.31)
	P value			0.16	0.82	0.53		0.24	0.71	0.57
ALC		162	107				117			
	Low	81	56	1 (reference)	1 (reference)	1 (reference)	61	1 (reference)	1 (reference)	1 (reference)
	High	81	51	0.63 (0.43-0.92)	0.67 (0.44-1.00)	0.61 (0.40-0.91)	56	0.64 (0.44-0.92)	0.68 (0.46-1.00)	0.62 (0.42-0.91)
	P value			0.02	0.05	0.02		0.02	0.05	0.02
ANC		162	107				117			
	Low	82	51	1 (reference)	1 (reference)	1 (reference)	56	1 (reference)	1 (reference)	1 (reference)
	High	80	56	1.28 (0.88-1.89)	1.22 (0.82-1.84)	1.16 (0.78-1.72)	61	1.28 (0.89-1.84)	1.22 (0.83-1.79)	1.15 (0.79-1.67)
	P value			0.20	0.33	0.47		0.20	0.32	0.47
NLR		162	107				117			
	High	82	62	1 (reference)	1 (reference)	1 (reference)	68	1 (reference)	1 (reference)	1 (reference)
	Low	80	45	0.44 (0.29-0.64)	0.41 (0.27-0.63)	0.40 (0.26-0.61)	49	0.43 (0.30-0.62)	0.43 (0.28-0.64)	0.41 (0.27-0.61)
	P value			<0.0001	<0.0001	<0.0001		<0.0001	<0.0001	<0.0001

Lymphocyte counts, ALC and ANC counts and NLR was divided into high and low group by the median split. The median of CD4$^+$, CD8$^+$, CD45RO$^+$ and FOXP3$^+$ T cells are 48, 140, 110 and 33 count/mm^2, respectively. The median of ALC and ANC were 1522 and 3251 count/mm^2, respectively. The median of NLR was 2.1.

[a] The hazard ratio was initially adjusted for age, sex, BMI, amount of alcohol consumption, family history of pancreatic cancer, history of smoking, diabetes mellitus, serum CA19-9, serum CEA

[b] The hazard ratio was initially adjusted for stage, histology, adjuvant, neo-adjuvant, residual tumor status, gene alterations (*KRAS, TP53, CDKN2A, SMAD4*).

ALC, absolute lymphocyte counts; ANC, absolute neutrophil counts; HR, hazard ratio; NLR, neutrophil-to lymphocyte ratio; PDAC, pancreatic ductal adenocarcinoma; TILs, tumor infiltrating lymphocytes.

Supplementary Table 6. Patient's characteristics according to adjuvant chemotherapy in PDAC

Characteristics	**Adjuvant chemotherapy**			***P* value**
	Absent (N=45)	**S-1** (N=74)	**GEM** (N=43)	
Age (years), Median (range)	72 (50-84)	69 (40-82)	65 (45-85)	0.007
BMI (kg/m^2), Median (range)	20.1 (16.5-27.6)	21.6 (14.3-33.2)	20.9 (14.9-31.2)	0.02
Sex				0.47
Male	23 (51.1%)	45 (60.8%)	22 (51.2%)	
Female	22 (48.9%)	29 (39.2%)	21 (48.8%)	
Family history of PDAC				0.35
Present	3 (6.7%)	5 (6.8%)	6 (14.0%)	
Absent	42 (93.3%)	69 (93.2%)	37 (86.0%)	
Alcohol consumption				0.09
< 50 (g/day)	37 (82.2%)	70 (94.6%)	39 (90.7%)	
≥ 50 (g/day)	8 (17.8%)	4 (5.4%)	4 (9.3%)	
History of smoking				0.006
Present	23 (51.1%)	43 (58.1%)	12 (27.9%)	
Absent	22 (48.9%)	31 (41.9%)	31 (72.1%)	
Diabetes mellitus				0.34
Present	20 (44.4%)	31 (41.9%)	13 (30.2%)	
Absent	25 (55.6%)	43 (58.1%)	30 (69.8%)	
CA19-9				0.58
< 37 (U/ml)	11 (24.4%)	16 (21.6%)	13 (30.2%)	
≥ 37 (U/ml)	34 (75.6%)	58 (78.4%)	30 (69.8%)	
CEA				0.41
< 5 (ng/ml)	30 (66.7%)	52 (70.3%)	34 (79.1%)	
≥ 5 (ng/ml)	15 (33.3%)	22 (29.7%)	9 (20.9%)	
Pathological Stage[a]				0.65
Ia/Ib	8 (17.8%)	19 (25.7%)	8 (18.6%)	
IIa/IIb	28 (62.2%)	36 (48.7%)	25 (58.1%)	
III	9 (20.0%)	19 (25.7%)	10 (23.3%)	
Histology grade				0.46
Well/Moderate	42 (93.3%)	64 (86.5%)	37 (86.1%)	
Poorly	3 (6.7%)	10 (13.5%)	6 (13.9%)	
Residual tumor status				0.003
R0	34 (75.6%)	61 (82.4%)	23 (53.5%)	
R1	11 (24.4%)	13 (17.6%)	20 (46.5%)	

(%) indicates the proportion of cases with specific features clinical or pathological characteristics according to adjuvant chemotherapy.

[a] Pathological stage was diagnosed based on TNM Classification of Malignant Tumors, 8th Edition.

BMI, body mass index; GEM, gemcitabine; PDAC, pancreatic ductal adenocarcinoma.