Supplementary Information of

Stress-induced phosphoprotein 1 as a secreted biomarker for human ovarian cancer promotes cancer cell proliferation

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Contents:

Supplementary Methods

Two versions of STIP1 ELISA

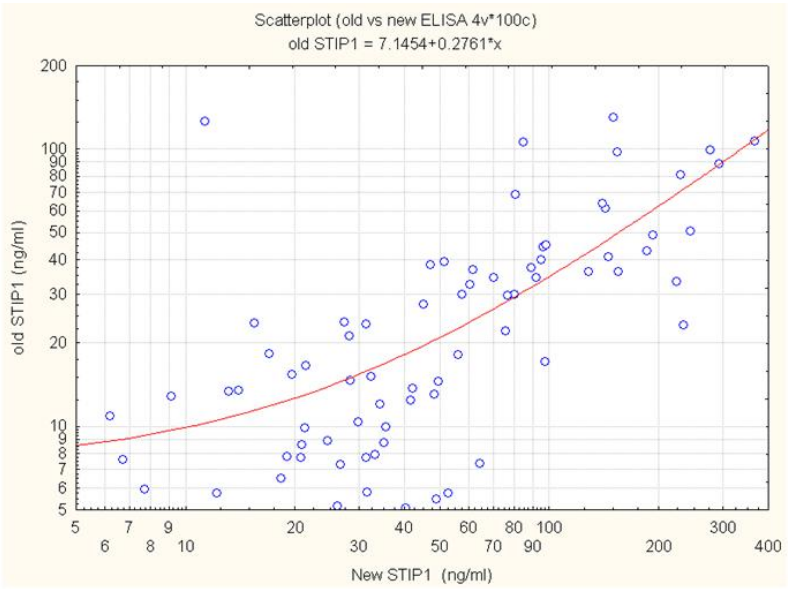
Originally, we developed an ELISA to determine serum levels of STIP1 using a chicken anti-human STIP1 polyclonal antibody (Abcam Laboratories, Cambridge, UK) as the capture antibody that was coated on 96-well plates (Nunc A/S, Roskilde, Denmark). Both human STIP1 recombinant protein and a mouse anti-human STIP1 monoclonal antibody were purchased from Abnova Corporation (Taipei, Taiwan). An HRP-conjugated sheep anti-mouse IgG antibody was purchased from Sigma-Aldrich (St Louis, MO), and the peroxidase substrate tetramethylbenzidine (TMB) was from Bethyl Laboratories (Montgomery, TX). However, after the supply of that chicken-origin antibody was discontinued for 6 months, the recent batch of that antibody did not work at all. Therefore, we had to develop the second version of the human STIP1 ELISA again.

Abnova Corporation (Taipei, Taiwan) has developed four monoclonal antibodies (MAb) against STIP1 protein, namely Clone 2E1, 2E11, 3A10 and 4H7. Each clone of antibody was further biotinylated for detection application. In our pilot study, 12 sandwich ELISA formats, each consisted of one MAb as capture Ab and another clone of biotinylated antibody as detecting Ab. The combination with highest signal at the highest point of the calibrator (100 ng/mL of STIP1 recombinant protein) and lowest background at the zero point was selected for final ELISA condition.

Briefly, ELISA plates (Nunc F8 MaxiSorp, A/S, Roskilde, Denmark) were coated with 2 μg/mL monoclonal antibodies (2E1, Abnova #H00010963-M33) in pH 9.6 carbonate-bicarbonate buffer at 4°C overnight and blocked with 250 μL/well of 0.05% Casein and 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at room temperature for 4 h. Calibrators, controls or patient serum and properly

diluted detection antibody (2E11, Abnova #H00010963-M35) were added simultaneously in coated well and incubated for 1h at 30°C. During the incubation period, a sandwich is formed consisting of solid phase antibody. After washing with 0.05% PBS-tween20, the Amdex streptavidin-peroxidase conjugated signal antibody was added (100 µL/well) and incubated for 1 h at 30°C. After washing, one hundred microtiters per well of TMB substrate was applied and color was developed for 10 min. The reaction was stopped by the addition of 100 µL/well of 1N H_2SO_4, and the color in the wells turned yellow. Absorbance was determined at 450 nm in a microplate spectrophotometer (Molecular Device SPECTRA Max model 190). The analyte concentrations were measured according to the calibration curve, for which recombinant human STIP1 was used as the calibrators. A calibration curve was prepared from 6 concentrations of calibrators, and the concentration of analyte in the serum specimen was determined by interpolation of 4-parameter curve fitting equation.

To compare whether the STIP1 values determined by the two versions of STIP1 ELISA were well correlated, we assayed the same batch of human sera (n=92) twice, each with one of these two ELISAs. Although different concentrations of STIP1 of the same specimen were derived from two versions of STIP1 ELISA, the results from these two ELISAs did show a good correlation, with R^2 at 0.455 ($P<0.000001$).

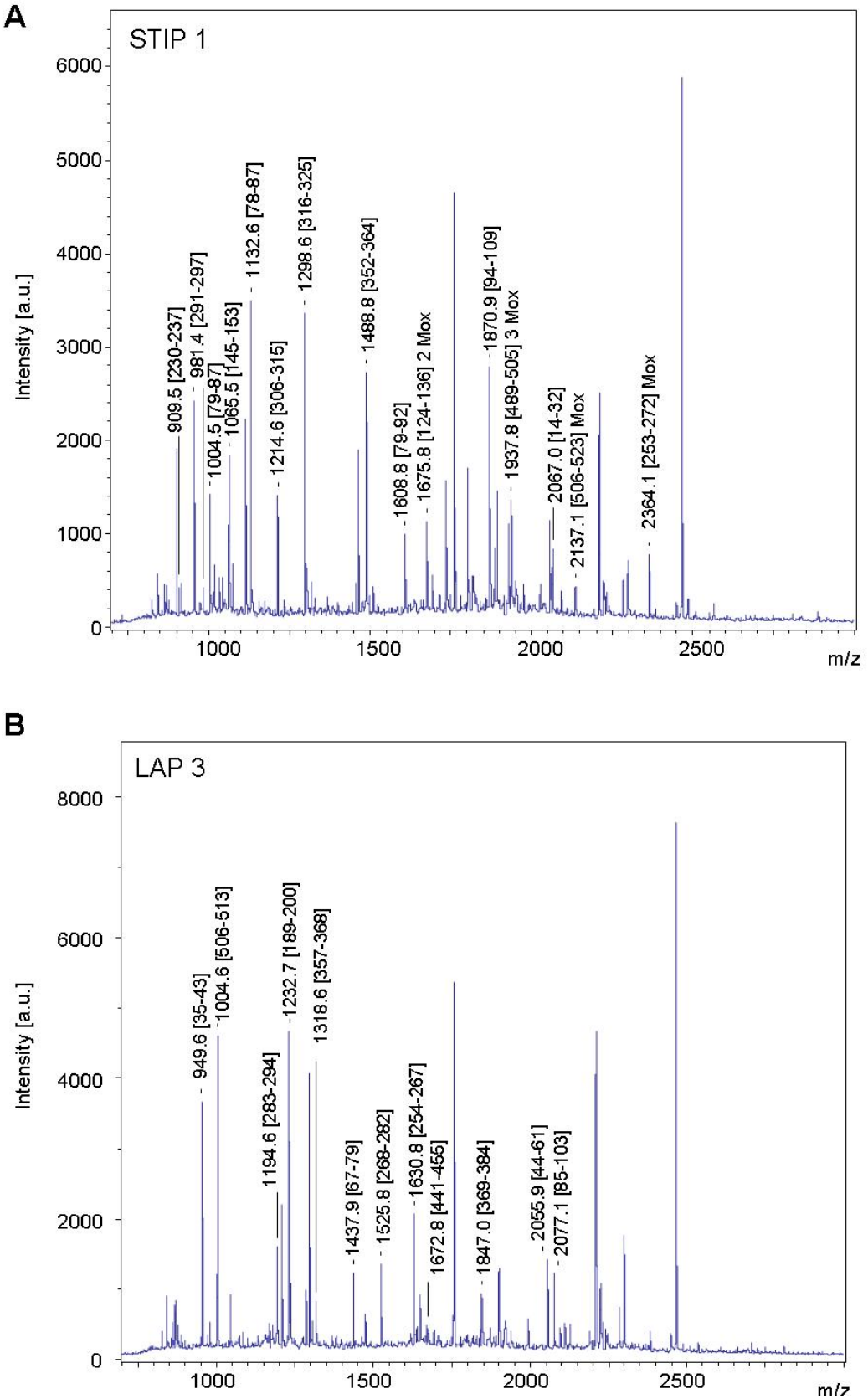


Multiple Regression Results

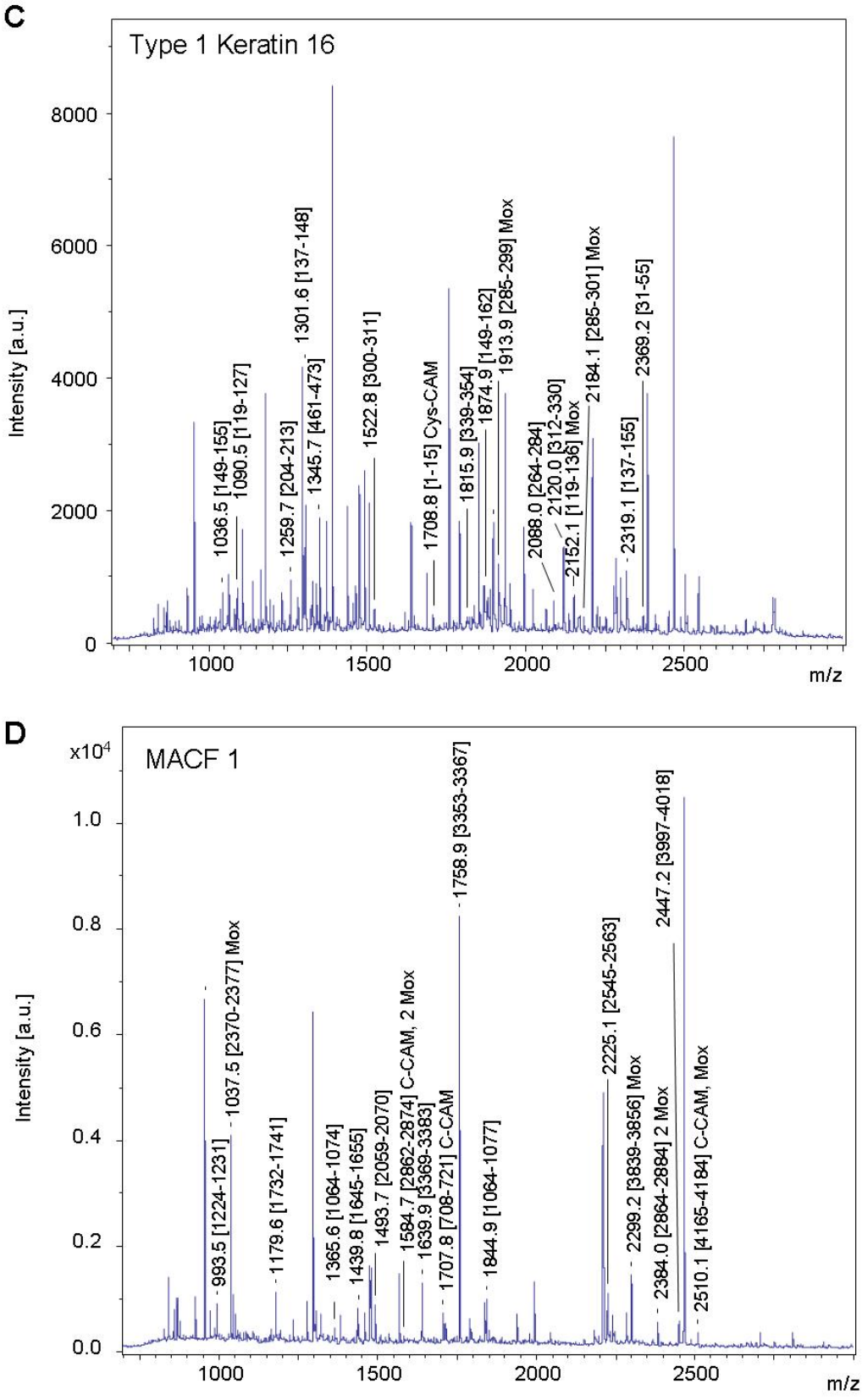
Dependent: old STIP1	Multiple R = .67431793	F = 75.04818	
	R2= .45470467	df = 1,90	
No. of cases: 92	adjusted R2= .44864583	p = .000000	
Standard error of estimate: 21.816833090			
Intercept: 7.145435787	Std.Error: 3.153177	t(90) = 2.2661	p = .0258

New STIP1 beta=.674

Supplementary Fig. 1. Annotated mass spectra of eight proteins (A-H)



Supplementary Fig. 1. Annotated mass spectra of eight proteins (A-H)



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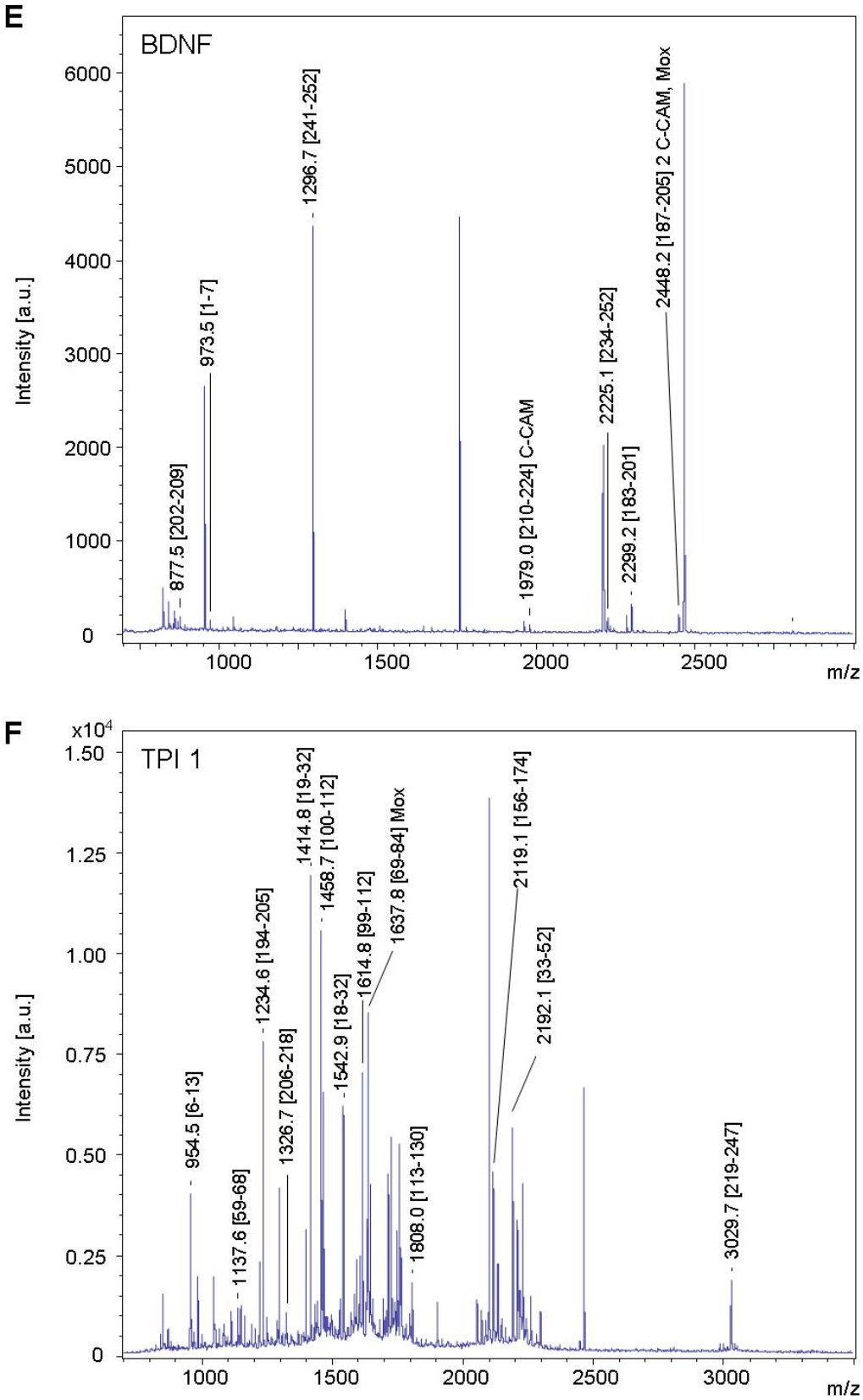
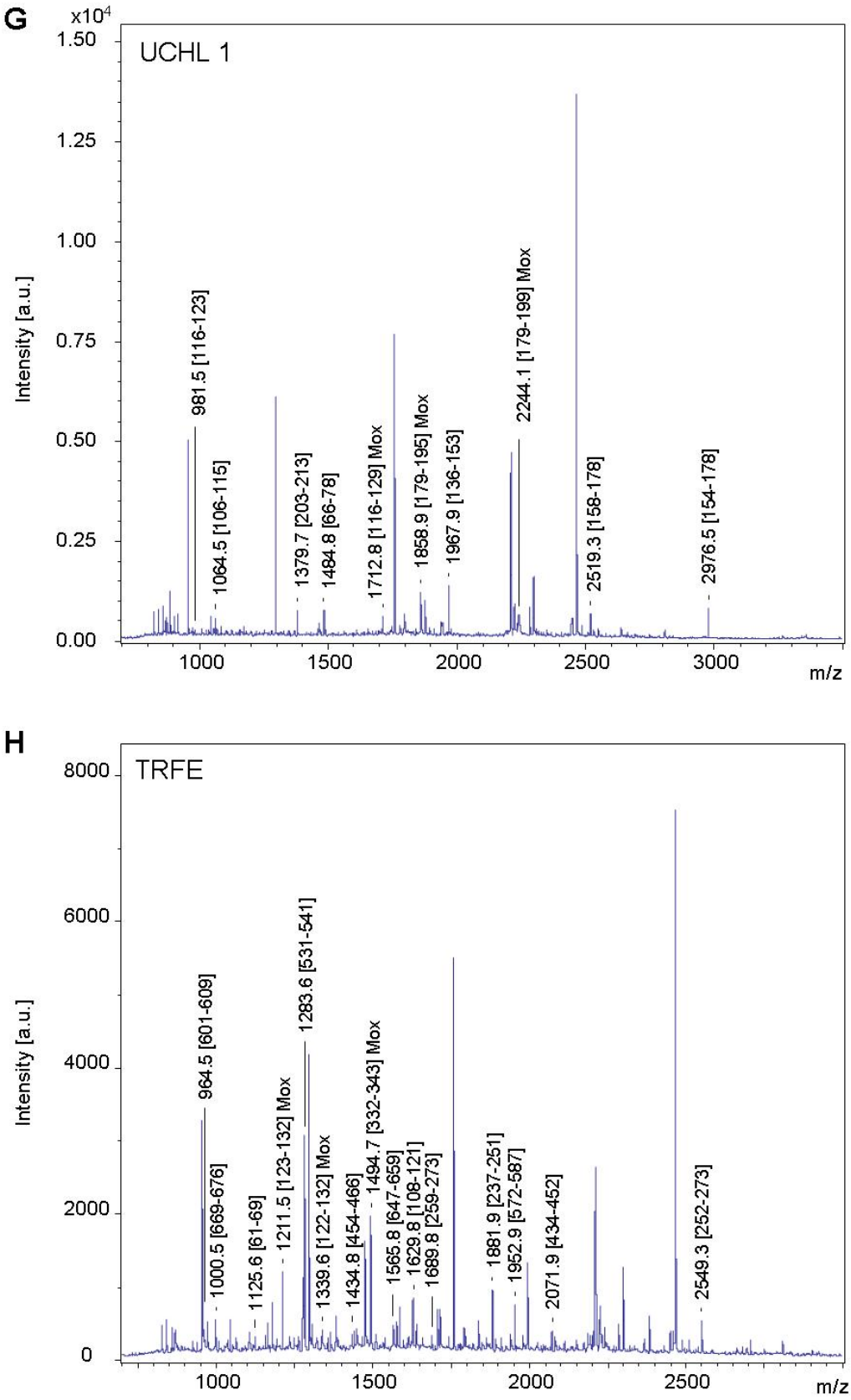
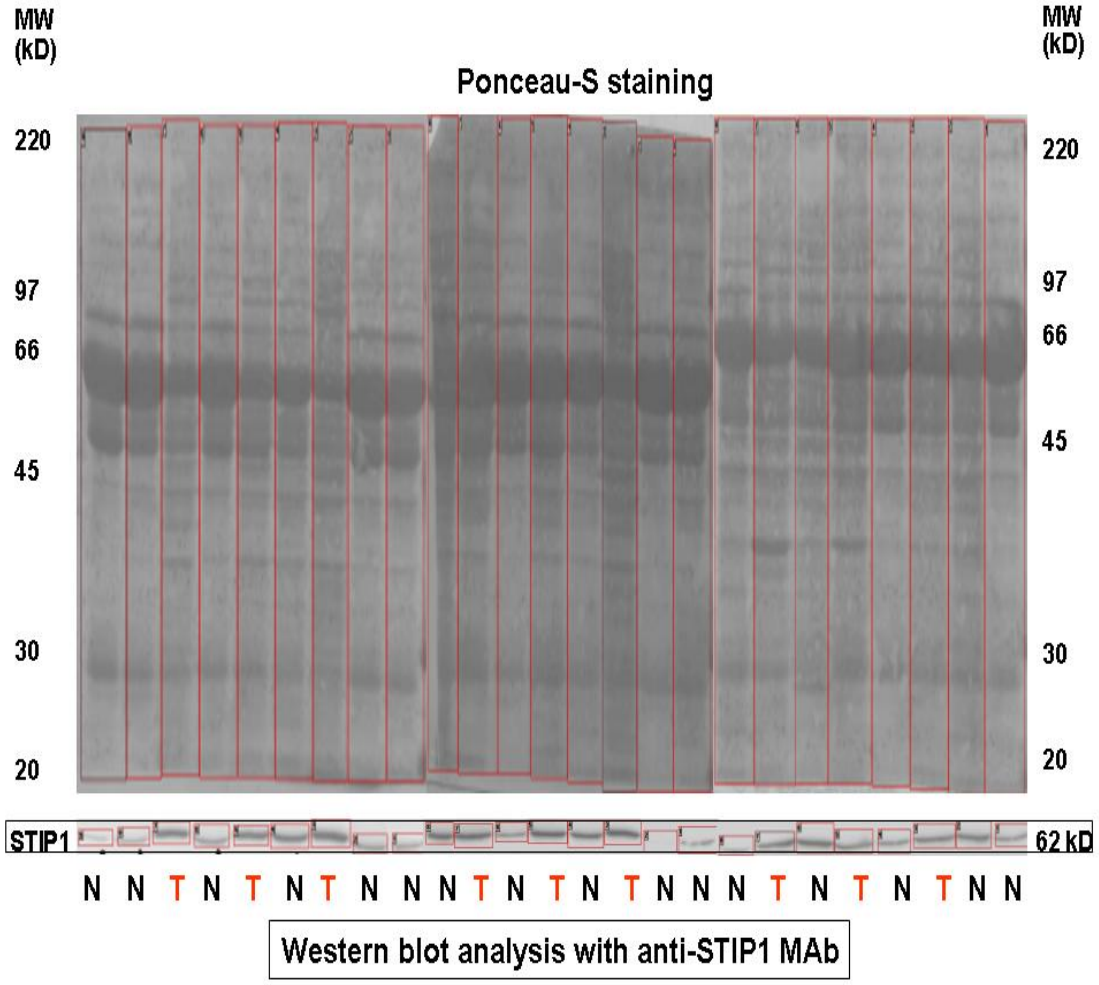


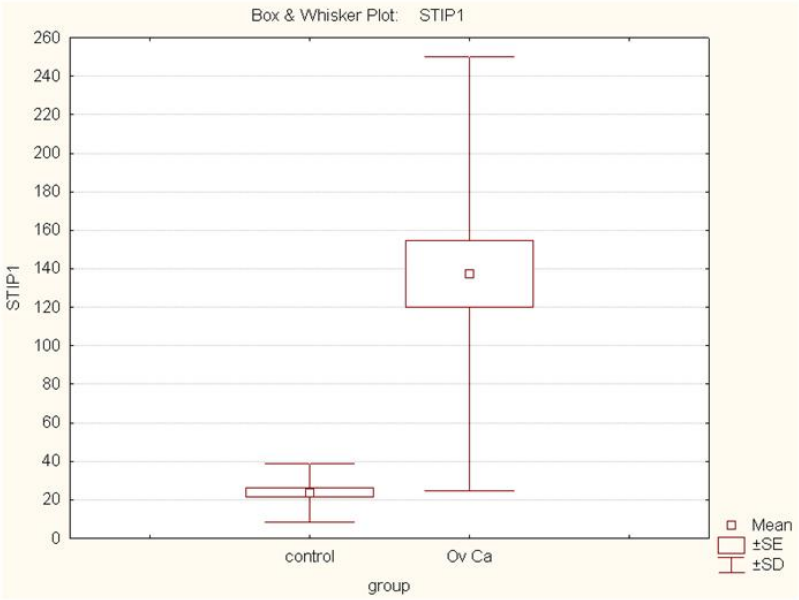
Supplementary Fig. 1. Annotated mass spectra of eight proteins (A-H)





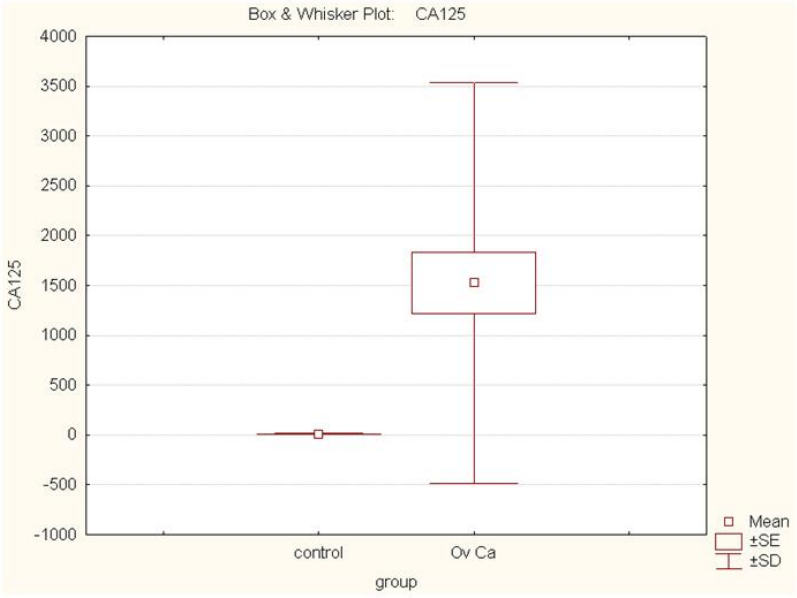
Supplementary Fig. 2.

Twenty-five protein specimens were electrophoresed in three 10% SDS-PAGE, and were subsequently transferred to three nitrocellulose membranes (9, 8, and 8 specimens for gels 1, 2, and 3, respectively). After nitrocellulose membranes were stained with Ponceau S (upper panel), protein contents of the membranes were digitalized by scanning and analyzed with *Unscan-it* software. The intensity of STIP1 in each lane by western blot analyses (lower panel) was normalized with the intensity of Ponceau S, thereby all 25 specimens could be compared.



T-tests; Grouping: group (CTR vs CA (2-19-2010))
Group 1: control
Group 2: Ov Ca

Variable	Mean control	Mean Ov Ca	t-value	df	p	Valid N control	Valid N Ov Ca	Std.Dev. control	Std.Dev. Ov Ca	F-ratio Variances	p Variances
STIP1	23.75581	137.3558	-6.55268	84	0.000000	43	43	15.09578	112.6757	55.71212	0.000000

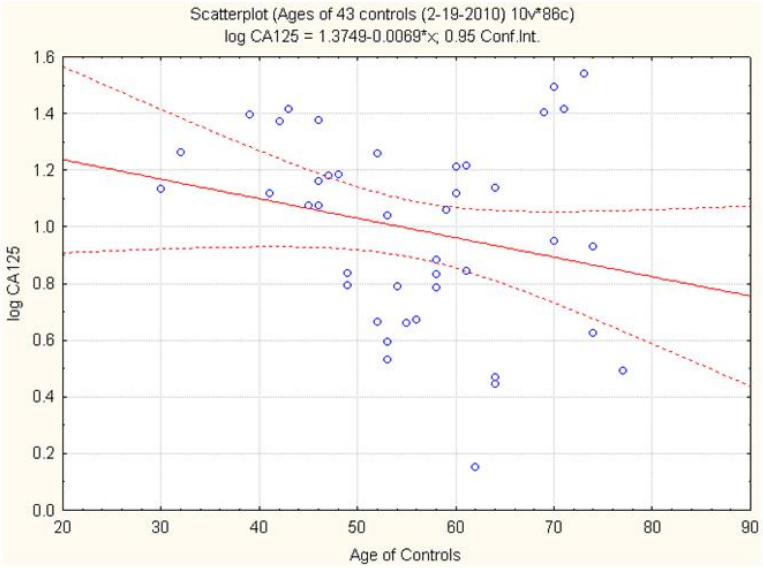


T-tests; Grouping: group (CTR vs CA (2-19-2010))
Group 1: control
Group 2: Ov Ca

Variable	Mean control	Mean Ov Ca	t-value	df	p	Valid N control	Valid N Ov Ca	Std.Dev. control	Std.Dev. Ov Ca	F-ratio Variances	p Variances
CA125	12.62000	1526.509	-4.92999	84	0.000004	43	43	8.440032	2013.626	56920.60	0.00

Supplementary Fig. 3. Statistics of serum levels of STIP1 (upper panel) and

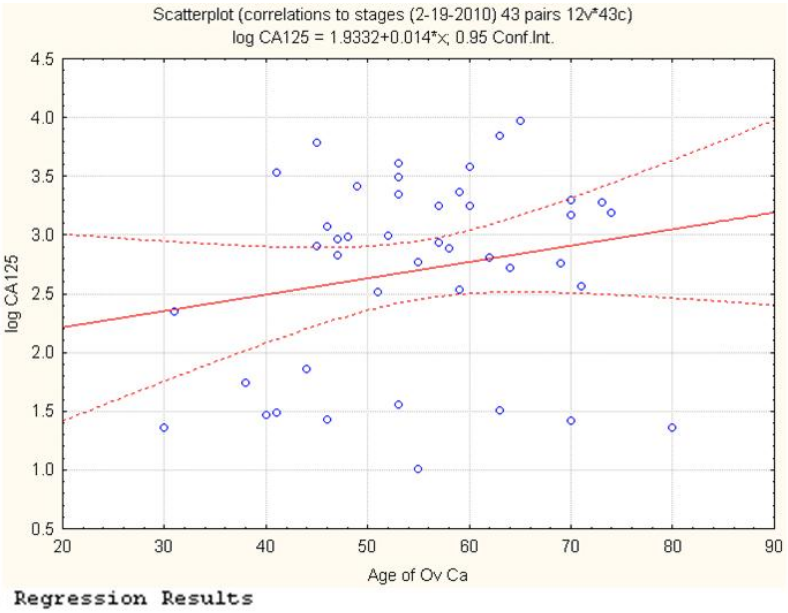
CA125 (lower panel) in the patients with ovarian cancer and age-matched controls. Significant differences in both STIP1 ($P<0.000001$) and CA125 ($P<0.00001$) were detected between controls (n=43, control) and patients with ovarian cancer (n=43, Ov Ca) using Statistica 6.1 (StatSoft Inc., Tulsa, OK).



Multiple Regression Results

Dependent: log CA125	Multiple R = .23838947	F = 2.470403	
	R2= .05682954	df = 1,41	
No. of cases: 43	adjusted R2= .03382538	p = .123694	
Standard error of estimate: .323588250			
Intercept: 1.374853445	Std.Error: .2476460	t(41) = 5.5517	p = .0000

Age of Contro beta=-.24

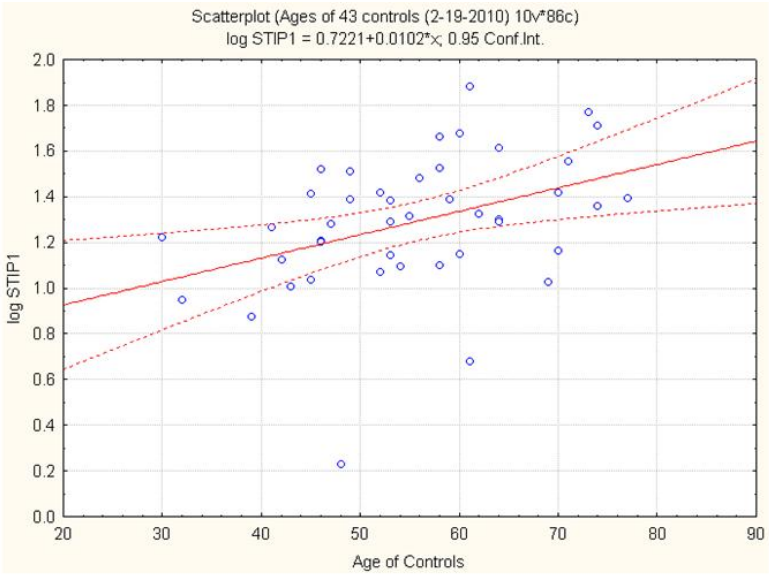


Multiple Regression Results

Dependent: log CA125	Multiple R = .20019245	F = 1.711760	
	R2= .04007702	df = 1,41	
No. of cases: 43	adjusted R2= .01666426	p = .198045	
Standard error of estimate: .808952609			
Intercept: 1.933214459	Std.Error: .6006677	t(41) = 3.2184	p = .0025

Age of Ov Ca beta=.200

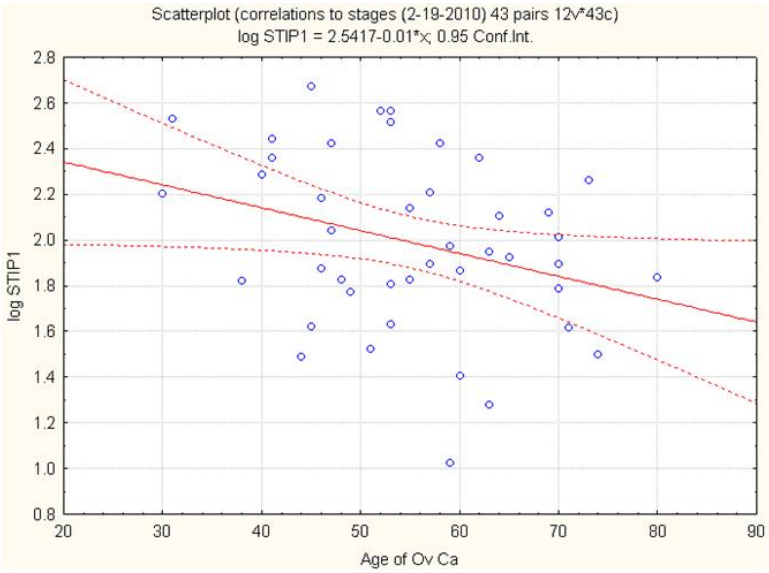
Supplementary Fig. 4. No correlation between ages and serum levels of CA125. Correlations between serum levels of CA125 and individual ages were calculated in 43 healthy controls (upper panel) and 43 patients with ovarian cancer (lower panel) using Statistica 6.1 (StatSoft Inc., Tulsa, OK). No correlation was found in either the healthy control group (P=0.124) or the ovarian cancer group (P=0.198). Detailed data are presented in **Supplementary Table 4**.



Multiple Regression Results

Dependent: log STIP1	Multiple R = .39143529	F = 7.418807	
	R2= .15322159	df = 1,41	
No. of cases: 43	adjusted R2= .13256846	p = .009441	
Standard error of estimate: .277783112			
Intercept: .722109388	Std.Error: .2125908	t(41) = 3.3967	p = .0015

Age of Contro beta=.391



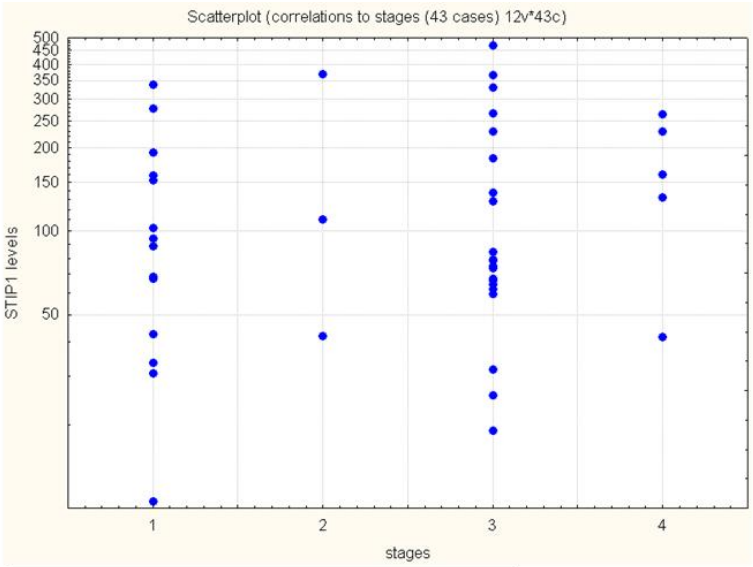
Multiple Regression Results

Dependent: log STIP1	Multiple R = .30799540	F = 4.296918	
	R2= .09486117	df = 1,41	
No. of cases: 43	adjusted R2= .07278461	p = .044507	
Standard error of estimate: .365871433			
Intercept: 2.541693886	Std.Error: .2716687	t(41) = 9.3559	p = .0000

Age of Ov Ca beta=-.31

Supplementary Fig. 5. Significant correlations between age and the serum levels of STIP1 in non-cancer controls and patients with ovarian cancer.

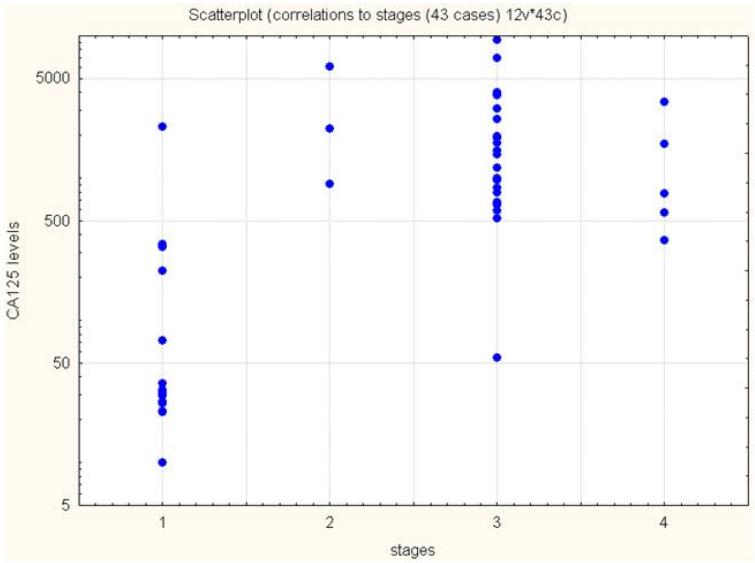
Correlations between serum levels of STIP1 and individual ages were calculated in 43 healthy controls (upper panel) and 43 patients with ovarian cancer (lower panel) using Statistica 6.1 (StatSoft Inc., Tulsa, OK). There were significant correlations in the healthy control group (P = 0.009) and in the ovarian cancer group (P= 0.045). Detailed data are presented in **Supplementary Table 4**.



LSD test; variable STIP1 levels (correlations to sta
Probabilities for Post Hoc Tests
Error: Between MS = 13345., df = 39.000

Cell No.	stages	{1} 118.66	{2} 173.43	{3} 137.96	{4} 165.52
1	1		0.460615	0.631068	0.440955
2	2	0.460615		0.621590	0.925749
3	3	0.631068	0.621590		0.634273
4	4	0.440955	0.925749	0.634273	

Stages of ovarian cancer vs. STIP1 levels



LSD test; variable CA125 levels (correlations to sta
Probabilities for Post Hoc Tests
Error: Between MS = 3357E3, df = 39.000

Cell No.	stages	{1} 252.15	{2} 3099.5	{3} 2185.8	{4} 1381.7
1	1		0.019204	0.004003	0.243812
2	2	0.019204		0.424002	0.206755
3	3	0.004003	0.424002		0.383147
4	4	0.243812	0.206755	0.383147	

Stages of ovarian cancer vs. CA125 levels

Supplementary Fig. 6. There was no significant differences in the serum levels of STIP1 (upper panel) among four stages of ovarian cancer. On the other hand, the CA125 levels in the stage I of ovarian cancer were significantly lower than those of stages II to III (lower panel).

Supplementary Table 1. Clinical information of the patients whose TIF was collected (n = 23). Only nine cases (marked as **Y**) of serous adenocarcinoma of the ovary were included in the study.

TIF number	**Age (y/o)**	**Pathology of the ovary**	**staging**	**Paired status**	**Inclusion in study**
T 1	74	Serous papillary adenocarcinoma	III	-	**Y**
T2	34	Endometrioid carcinoma, grade 1-2	IIIa	-	no
T3	67	Endometrioid carcinoma, poorly differentiated	III	-	no
T4	81	Serous adenocarcinoma, poorly differentiated	IV	-	**Y**
T5	71	Serous papillary adenocarcinoma	IIIc	-	**Y**
T6	73	Fibroma and thecoma	benign	-	no
T7	43	Serous papillary adenocarcinoma	IIIc	-	**Y**
T8	49	Serous adenocarcinoma with focal clear cell carcinoma	IIIc	-	no
T9	84	Serous adenocarcinoma	IIc	-	**Y**
T10	48	Mucinous adenocarcinoma, grade 1	Ia	-	no
T11	41	Endometrioid carcinoma, well-differentiated	IIc	-	no
T12	65	Serous papillary adenocarcinoma	IIc	-	**Y**
T13	51	Serous papillary adenocarcinoma	IIc	Paired with N19	**Y**
T14	31	Immature teratoma	III	-	no
T15	49	Endometrioid adenocarcinoma, well-differentiated	Ib	-	no
T16	79	Mucinous tumor, borderline	-	-	no

T17	22	Mucinous adenocarcinoma, moderate-differentiated	IIIc	-	no
T18	33	Mucinous tumor, borderline (grade 1)	Ia	-	no
T19	43	Adenocarcinoma, high grade	IIIc	Paired with N16	no
T20	45	Serous papillary carcinoma	IV	-	**Y**
T21	31	Serous adenocarcinoma	IIc	-	**Y**
T22	55	Clear cell carcinoma	IIIc	Paired with N18	no
T23	54	Mucinous cystadenoma	-	Paired with N17	no

Supplementary Table 2. Clinical information of the patients whose NIF was collected (n = 19). Bilateral ovaries were negative for malignancy, except for three paired cases whose unilateral ovary was negative for malignancy. Sixteen cases of pathology-free ovaries were included in the study.

NIF number	Age (y/o)	Pathology of the disease	Staging (if applicable)	Paired status	Inclusion in study
N1	49	Uterine myoma	-	-	**Y**
N2	40	Cervical carcinoma	Ib	-	**Y**
N3	37	Endometrial carcinoma	Ib	-	**Y**
N4	60	Endometrial carcinoma	Ic	-	**Y**
N5	52	Endometrial carcinoma	Ia	-	**Y**
N6	54	Endometrial carcinoma	IV	-	**Y**
N7	37	Endometrial carcinoma	IIa	-	**Y**
N8	46	Endometrial carcinoma	II	-	**Y**
N9	40	adenomyosis	-	-	**Y**
N10	49	Endometrial carcinoma (well differentiated)	-	-	**Y**
N11	49	CIN3 of the cervix	-	-	**Y**
N12	50	Cervical cancer	Ib2	-	**Y**
N13	52	Uterine carcinosarcoma	IIIc	-	**Y**
N14	48	Adenocarcinoma of the cervix	Ib1	-	**Y**
N15	45	Squamous cell carcinoma of the cervix (poorly differentiated)	Ib	-	**Y**
N16	43	Adenocarcinoma of left ovary, right ovary was free for malignancy	IIIc	Paired with T19	no
N17	54	Mucinous cystadenoma of right ovary, left ovary was free for tumor	-	Paired with T23	**Y**
N18	55	Clear cell carcinoma of right ovary, left ovary was free of malignancy	IIIc	Paired with T22	no
N19	51	Serous papillary adenocarcinoma of right ovary, left ovary was free for malignancy	IIc	Paired with T13	no

Supplementary Table 3. Protein identification by peptide fingerprint.

Protein Name	Gene name	Accession Number	MASCOT Score	sequence coverage (%)	# of mateched peaks	# of unmateched peaks
Stress-induced-phosphoprotein 1	STIP1	gil5803181	190	44	26	46
Leucine aminopeptidase	LAP3	gil4335941	200	48	21	45
Type I keratin 16	Type I Keratin 16	gil1195531	75	53	26	110
Macrophin 1 isoform 2	MACF1	gil17426164	76	14	53	32
Brain-derived neurotrophic factor isoform b preproprotein	BDNF	gil25306235	67	35	9	18
Triosephosphate Isomerase	TPI 1	gil999892	172	85	20	112
Ubiquitin carboxyl-terminal esterase L1	UCHL1	gil21361091	138	58	12	66
Transferrin	TRFE	gil4557871	147	33	23	59

Supplementary Table 4. Detailed clinical information and serum concentrations of CA125 and STIP1 in forty three pairs of age-matched ovarian cancer patients and healthy controls

Cancer No.	Age	Cancer Stage	Histopathology	CA125	STIP1 ng/mL	Normal No.	Age	CA125	STIP1 ng/mL
Ca 1	53	I B	serous adenocarcinoma	36.2	42.6	N 1	53	3.4	13.9
Ca 2	71	IV	serous adenocarcinoma	366.6	41.6	N 2	71	26.2	36.1
Ca 3	70	III C	serous adenocarcinoma	1965.3	78.5	N 3	70	31.2	26.2
Ca 4	63	III B	mixed clear cell and serous adenocarcinoma	6979.3	19.0	N 4	64	13.8	19.9
Ca 5	53	III C	mucinous adenocarcinoma	4043.4	64.5	N 5	53	11.0	19.6
Ca 6	55	III C	serous adenocarcinoma	593.8	138.3	N 6	55	4.6	20.7
Ca 7	46	III C	serous adenocarcinoma	1190.9	74.8	N 7	46	14.5	16.1
Ca 8	74	III C	adenocarcinoma	1556.9	31.6	N 8	74	8.5	51.4
Ca 9	58	IV	serous adenocarcinoma	778.7	264.2	N 9	58	7.7	12.6
Ca 10	31	I C	endometrioid adenocarcinoma with mucinous differentiation	225.3	338.3	N 10	32	18.3	8.9
Ca 11	60	III C	serous adenocarcinoma	1774.0	73.3	N 11	60	13.2	47.7
Ca 12	38	III C	papillary serous adenocarcinoma	54.8	66.5	N 12	39	25.0	7.5
Ca 13	57	IV	papillary serous adenocarcinoma	1758.0	160.8	N 13	58	6.8	33.6
Ca 14	59	I C	clear cell carcinoma	343.2	94.5	N 14	59	11.5	24.5
Ca 15	41	IV	serous adenocarcinoma	3433.0	228.6	N 15	43	26.1	10.2
Ca 16	60	III C	serous adenocarcinoma	3815.8	25.6	N 16	61	16.5	76.7
Ca 17	47	III C	clear cell adenocarcinoma	680.4	266.3	N 17	47	15.2	19.2
Ca 18	45	III C	serous adenocarcinoma	800.0	469.2	N 18	45	11.9	26
Ca 19	53	II A	serous adenocarcinoma	2242.9	368.2	N 19	54	6.2	12.5
Ca 20	73	III C	serous adenocarcinoma	1900.2	183.5	N 20	73	34.9	59.1

Ca 21	51	I C	serous adenocarcinoma	331.5	33.4	N 21	52	18.2	11.8
Ca 22	48	III C	serous adenocarcinoma	974.0	67.2	N 22	49	6.3	32.3
Ca 23	80	I C	mucinous adenocarcinoma	22.8	68.8	N 23	77	3.1	24.7
Ca 24	57	III C	serous adenocarcinoma	854.9	79.0	N 24	58	6.1	46.1
Ca 25	53	III B	clear cell carcinoma	3087.9	330.1	N 25	53	3.9	24.2
Ca 26	69	IV	serous adenocarcinoma	572.2	132.4	N 26	69	25.3	10.6
Ca 27	59	I C	serous adenocarcinoma	2319.6	10.6	N 27	60	16.3	14.1
Ca 28	44	I C	clear cell adenocarcinoma	72.3	31	N 28	46	11.9	16
Ca 29	47	II C	tubal cancer	919.6	110	N 29	48	15.4	1.7
Ca 30	49	III C	serous adenocarcinoma	2613.1	59	N 30	49	6.9	24.5
Ca 31	45	II A	endometrioid adenocarcinoma	6136.0	42	N 31	46	23.8	33.1
Ca 32	62	III C	papillary serous adenocarcinoma	647.0	229.1	N 32	64	3.0	19.6
Ca 33	70	III C	papillary serous adenocarcinoma	1476.0	61.6	N 33	74	4.2	22.8
Ca 34	65	III C	papillary serous adenocarcinoma	9376.0	84.5	N 34	64	2.8	41.0
Ca 35	64	III C	serous adenocarcinoma	524.4	128.2	N 35	62	1.4	21.2
Ca 36	52	III C	serous adenocarcinoma	994.7	367.0	N 36	52	4.6	26.1
Ca 37	55	I A	borderline mucinous tumor	10.1	67.5	N 37	56	4.7	30.4
Ca 38	70	I B	adenocarcinoma, mixed clear cell and serous types	26.2	102.7	N 38	70	8.9	14.6
Ca 39	41	I A	mucinous cystadenocarcinoma	31.1	278.3	N 39	41	13.2	18.5
Ca 40	40	I A	mucinous cystadenocarcinoma	29.4	192.8	N 40	42	23.6	13.3
Ca 41	46	I A	borderline mucinous tumor	27.1	153.2	N 41	45	11.9	10.9
Ca 42	30	I B	borderline mucinous tumor	23.1	159.1	N 42	30	13.7	16.8
Ca 43	63	I A	mucinous adenocarcinoma	32.2	88.7	N 43	61	7.0	4.8