Supplemental materials

Methods

Patients with esophageal benign disease

The detail of 44 patients with esophageal benign disease in test cohort: 17 cases of esophageal leiomyoma**,** 11 cases of upper gastrointestinal polyps, 4 cases of esophageal cyst, 4 cases of esophageal papilloma, 3 cases of low grade atypical hyperplasia, 2 cases of esophageal varices, 1 cases of reflux esophagitis, 1 cases of esophageal gastric mucosal endometriosis, and 1 cases of mycoderma thickening. 74 patients with esophageal benign disease in the validation cohort containing 27 cases of reflux esophagitis, 10 cases of Barrett's esophagus, 5 cases of esophageal cyst, 2 cases of low grade atypical hyperplasia, 5 cases of esophageal gastric mucosal endometriosis, 2 cases of esophageal varices, 9 cases of esophageal papilloma and 14 cases of esophageal leiomyomas.

RNA isolation, cDNA library preparation, RNA-seq and analysis

Total RNA was extracted from frozen tissues using the Trizol reagent (Invitrogen, USA) according to the manufacture’s instruction. Beads with oligo(dT) were used to isolate poly(A) mRNA. First-strand cDNA was synthesized using random hexamer-primer and reverse transcriptase (Invitrogen). The second-strand cDNA was synthesized using RNase H (Invitrogen) and DNA polymerase I (New England BioLabs). Then the cDNA libraries were prepared according to Illumina's protocols

and sequenced by Illumina HiSeq™ 2000. Sequence data from genomic DNA and complementary DNA were mapped to the reference human genome (hg19) using the Burrows-Wheeler Aligner and were processed using the publicly available SAMtools, Picard, and Genome Analysis Toolkit. The quantity of gene expression was calculated by the RPKM method(Reads Per Kb per Million reads)[1]. The genes with FDR less 0.001 and change fold more than 2 fold were considered as the DEG (Differentially expressed gene).

Immunohistochemistry

Formalin-fixed, paraffin-embedded ESCC sections were incubated with antibodies to CHI3L1(Abcam, UK), MMP13(R&D systems, USA), SPP1(Abcam, UK) overnight at 4°C. After washing in PBST, the tissue sections were treated with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:1000, Zymed). The tissue sections were then developed with 3-diaminobenzidine tetrahydrochloride for 10 seconds, followed by counterstaining with 10% Mayer's hematoxylin. The degree of immunostaining was reviewed by two independent observers.

Results

Cut-off values

As shown in Table S5, for each stage, to reach 90% sensitivity, the cut-off values for CHI3L1, MMP13, and SPP1 in the test cohort were 25.19, 0.62 and 19.08 in stage I; 27.15, 0.43 and 19.87 in stage II; 42.78, 0.61 and 32.74 in stage III; and 42.29, 0.30

and 31.78 in stage IV, respectively. In the validation cohort, meanwhile, the breakpoints for CHI3L1, MMP13, and SPP1 were 31.37, 0.58 and 12.48 in stage I; 33.49, 0.70 and 13.08 in stage II; 34.62, 0.41 and 12.38 in stage III; and 34.01, 0.66 and 15.55 in stage IV, respectively.

The comparison between CHI3L1, MMP13, or SPP1 combination and CEA

In comparison to the traditional ESCC marker CEA, as shown in Figure S6A and S6B, the AUC of CEA was 0.646 (95% CI: 0.583–0.710) in the test cohort and 0.633 (95% CI: 0.573–0.694) in the validation cohort. Moreover, when we applied 5.0 ng/ml, according to the manufacturer's protocol, for CEA as the cut-off values, as shown in Figure S6B, the sensitivity of CEA was 6.7% and 13.0% in the test and validation cohort, respectively, which is significantly lower than that for the combination (90.00%), which was rather low to apply for the early detection of ESCC. However, the specificity was slightly higher. Moreover, the combination exhibited a higher NPV compared with CEA (87.07% vs. 48.91% in the test cohort, and 93.52% vs. 49.83% in the validation cohort) without an obvious change in the PPV (77.59% vs. 62.5% in the test cohort, and 62.14% vs. 73.33% in the validation cohort).

The association between serum CHI3L1, MMP13 and SPP1 and clinicopathological characteristics

The associations between the median serum CHI3L1, MMP13, SPP1 levels and the clinicopathological parameters are presented in Table S6. Serum CHI3L1 and MMP13 was not significantly correlated with gender, T classification, N classification, metastasis, or clinical stage, but it was significantly associated with age ($P = 0.0002$

and $P = 0.0267$, respectively). The serum level of CHI3L1 was higher in elderly patients (≥60 years) than in patients aged less than 60 years, whereas the serum level of MMP13 was lower in elderly patients (≥60 years) than in patients aged less than 60 years. Serum SPP1 was not significantly correlated with age, N classification, metastasis, or clinical stage, but it was correlated with gender and T classification (P = 0.0037 and P =0.0165, respectively). The level of serum SPP1 was elevated in male compared with female patients, and T4 patients exhibited the highest level of SPP1.

References:

[1] Mortazavi A, Williams BA, McCue K, et al. Mapping and quantifying mammalian transcriptomes by RNA-Seq[J]. Nat Methods. 2008,5(7)：621-628.

[2] Bilgin DE, Dizdar Y, Aksit E, et al. EMMPRIN and ADAM12 in prostate cancer: Preliminary results of a prospective study[J]. Tumour Biol. 2014,35(11)：11647-11653.

[3] Sole X, Crous-Bou M, Cordero D, et al. Discovery and validation of new potential biomarkers for early detection of colon cancer[J]. PLoS One. 2014,9(9)：e106748.

[4] Tanwar MK, Gilbert MR, Holland EC. Gene expression microarray analysis reveals YKL-40 to be a potential serum marker for malignant character in human glioma[J]. Cancer Res. 2002,62(15)：4364-4368.

[5] Johansen JS, Christensen IJ, Riisbro R, et al. High serum YKL-40 levels in patients with primary breast cancer is related to short recurrence free survival[J]. Breast Cancer Res Treat. 2003,80(1)：15-21.

[6] Zheng X, Xing S, Liu XM, et al. Establishment of using serum YKL-40 and SCCA in combination for the diagnosis of patients with esophageal squamous cell carcinoma[J]. Bmc Cancer. 2014,14：490.

[7] Yoneda K, Iida H, Endo H, et al. Identification of Cystatin SN as a novel tumor marker for colorectal cancer[J]. Int J Oncol. 2009,35(1)：33-40.

[8] Kosanam H, Prassas I, Chrystoja CC, et al. Laminin, gamma 2 (LAMC2): A promising new putative pancreatic cancer biomarker identified by proteomic analysis of pancreatic adenocarcinoma tissues[J]. Mol Cell Proteomics. 2013,12(10)：2820-2832.

[9] Jiao XL, Chen D, Wang JG, et al. Clinical significance of serum matrix metalloproteinase-13 levels in patients with esophageal squamous cell carcinoma (ESCC)[J]. Eur Rev Med Pharmacol Sci. 2014,18(4)：509-515.

[10] Etoh T, Inoue H, Yoshikawa Y, et al. Increased expression of collagenase-3 (MMP-13) and MT1-MMP in oesophageal cancer is related to cancer aggressiveness[J]. Gut. 2000,47(1)：50-56.

[11] Sasaki H, Dai M, Auclair D, et al. Serum level of the periostin, a homologue of an insect cell adhesion molecule, in thymoma patients[J]. Cancer Lett. 2001,172(1)：37-42.

[12] Fersching DM, Nagel D, Siegele B, et al. Apoptosis-related biomarkers sFAS, MIF, ICAM-1 and PAI-1 in serum of breast cancer patients undergoing neoadjuvant chemotherapy[J]. Anticancer Res. 2012,32(5)：2047-2058.

[13] Shih YL, Hsieh CB, Lai HC, et al. SFRP1 suppressed hepatoma cells growth through Wnt canonical signaling pathway[J]. Int J Cancer. 2007,121(5)：1028-1035.

[14] Mor G, Visintin I, Lai Y, et al. Serum protein markers for early detection of ovarian cancer[J]. Proc Natl Acad Sci U S A. 2005,102(21)：7677-7682.

Figure S1. 175 genes differently expressed in at least 5 out of 6 tissue pairs form RNA sequencing. Red:upregulated genes; Green:downregulated genes. The X axis is measured by log2(T/N). T:ESCC tissue, N:normal tissue.

Figure S2. 39 differently expressed genes that were upregulated by at least 5-fold in 4 out of 6 tumor tissues compared to the non-cancerous tissues. The X axis is measured by log2(T/N). T:ESCC tissue, N:normal tissue.

Figure S3. 32 secretory proteins identified through SignalP4.1 and SecretomeP 2.0.

Figure S4. The expression level of 32 candidates in three PubMed GEO database.

A.GSE23400(53N,53T); B.GSE20347(17N,17T), C.GSE33810(GSM836193: mixture of 10 normal tissues; GSM836194: mixture of 10 ESCC tissues). N:normal tissue, T:ESCC tissue. The X axis is measured by log2(T/N).

Figure S5. Serum levels of candidate biomarkers in the preliminary screening phase.

Levels of serum CA9, CST1, LAMC2, POSTN, SERPINE1, and SFRP4, were compared between 40 ESCC patients (ESCC) and 40 healthy controls (HC). The Mann-Whitney U test was performed for comparisons between groups. $P < 0.05$ was considered statistically significant.

Figure S6. Diagnostic effect of serum CEA in the test cohort and the validation cohort.

A: The comparision of ROC curves for CEA and the combination (Logit(p=ESCC)=-4.583+0.017×CHI3L1+0.018×SPP1+0.821×MMP13) in discriminating patients with 150 ESCC from 140 controls. B: The comparision of ROC curves for CEA and the combination in discriminating patients with169 ESCC from 154 controls. C: The diagnostic performance of CEA in discriminating ESCC and controls (healthy controls and patients with esophageal benign disease) in the test

cohort and validation cohort. ESCC:esophageal squamous cell carcinoma; Sen, sensitivity; Spe, specificity; PPV, positive predictive value; NPV, negative predictive value; HC,healthy control.

Table S1. Primers for real-time RT-PCR

Gene	primer	length（bp）
GAPDH pF	5′-GACTCATGACCACAGTCCATGC-3′	113
GAPDH pR	5′-AGAGGCAGGGATGATGTTCTG-3′	
CHI3L1 pF	5’- GAGGATGGAACTTTGGGTCTC -3’	182
CHI3L1 pR	5’- TCATTTCCTTGATTAGGGTGGT -3’	
MMP13 pF	5’-CCAAGGACCCTGGAGCACTC-3’	173
MMP13 pR	5’-CAAGGGATAAGGAAGGGTCAC-3’	
SPP1 pF	5’-GACCTGCCAGCAACCGAAG-3’	155
SPP1 pR	5’-GGTGATGTCCTCGTCTGTAGC-3’	

Table S2. Pathway analysis of significantly differentially expressed genes.

Markers	Pathway
ADAM12	EFGR,NOTCH
CA9	Angiogenesis; Hypoxia
CHI3L1	NF-KB
COL11A1	PI3K-Akt
CST1	Wnt
CTHRC1	Wnt

INHBA	TGF-β
LAMC2	PI3K-Akt
MFAP2	NOTCH
MMP13	Degradation of the extracellular matrix
POSTN	NOTCH
SERPINE1	P53
SFRP4	Wnt
SPP1	PI3K-Akt
WISP1	NOTCH Wnt
HMGA2	Transcriptional misregulation in cancer
HOXD10	Stem Cell Transcription Factors in human, Proteoglycans in cancer, MicroRNAs in cancer
SIX1	Wnt; Transcriptional misregulation in cancer

Table S3. GO Biological process analysis of significantly differentially expressed genes.

Markers	GO Biological process
ADAM12	cell adhesion
CA9	response to hypoxia
CHI3L1	activation of NF-kappaB-inducing kinase activity
COL11A1	extracellular matrix organization
CST1	negative regulation of endopeptidase activity
CTHRC1	cell migration

INHBA	negative regulation of cell cycle, extrinsic apoptotic signaling pathway, cell differentiation, growth
LAMC2	cell junction assembly, cell adhesion
MFAP29	extracellular matrix organization
MMP13	extracellular matrix organization
POSTN	cell adhesion
SERPINE1	angiogenesis
SFRP4	negative regulation of cell proliferation
SPP1	cell adhesion
WISP1	regulation of cell growth
HMGA2	cell proliferation, epithelial to mesenchymal transition
HOXD10	epithelial to mesenchymal transition
SIX1	apoptotic process

Table S4. Evidence sourced from literature about markers existence in blood.

Markers	Evidence in literature
ADAM12	Prostate cancer[2]
CA9	Breast cancer[3]
CHI3L1	Melanoma,[4] breast cancer,[5] ESCC[6]
CST11	Colon cancer[7]
LAMC2	Pancreatic cancer[8]
MMP13	ESCC[9, 10]
POSTN	Thymoma[11]

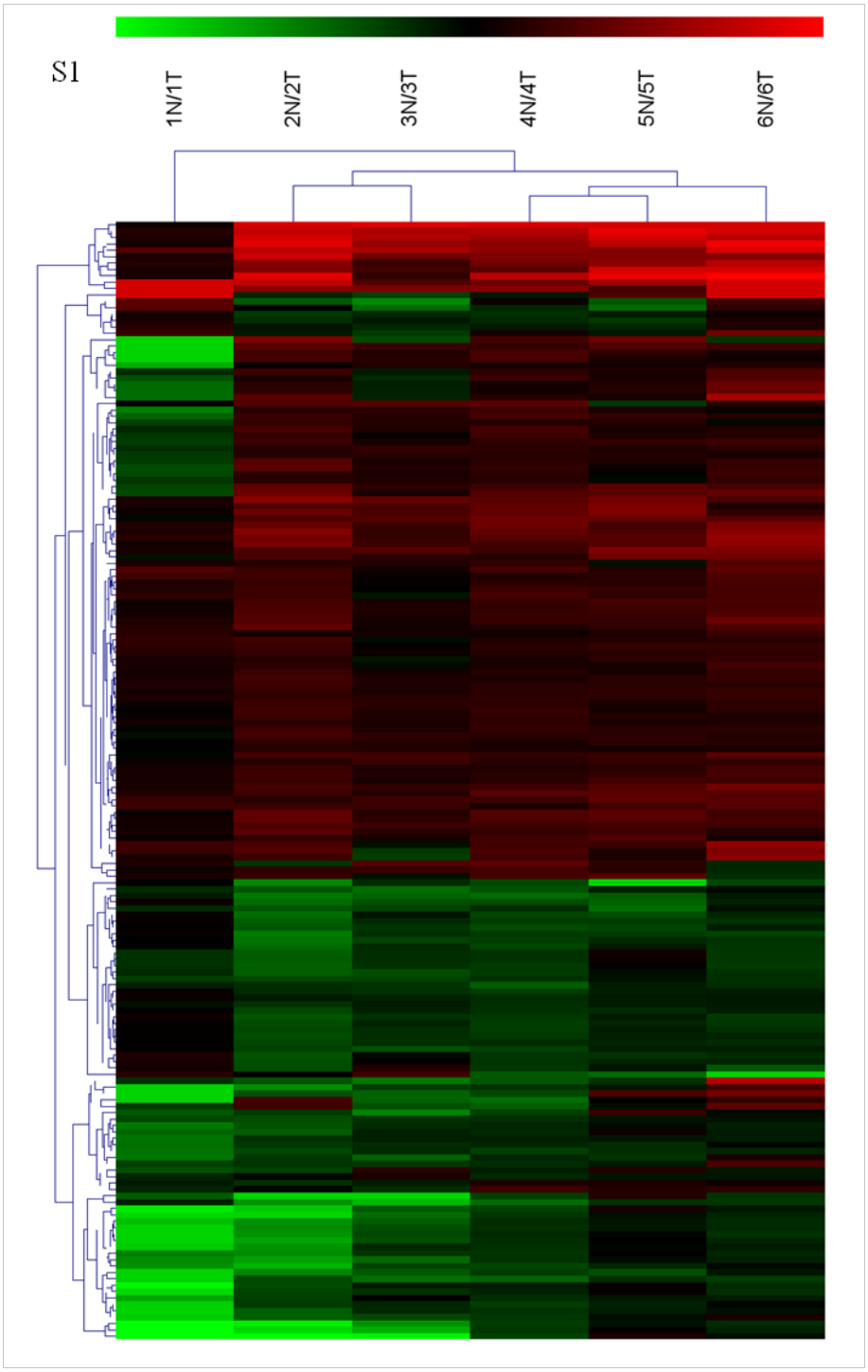
SERPINE1	Breast cancer[12]
SFRP4	Hepatocellular carcinoma[13]
SPP1	ESCC[13, 14]

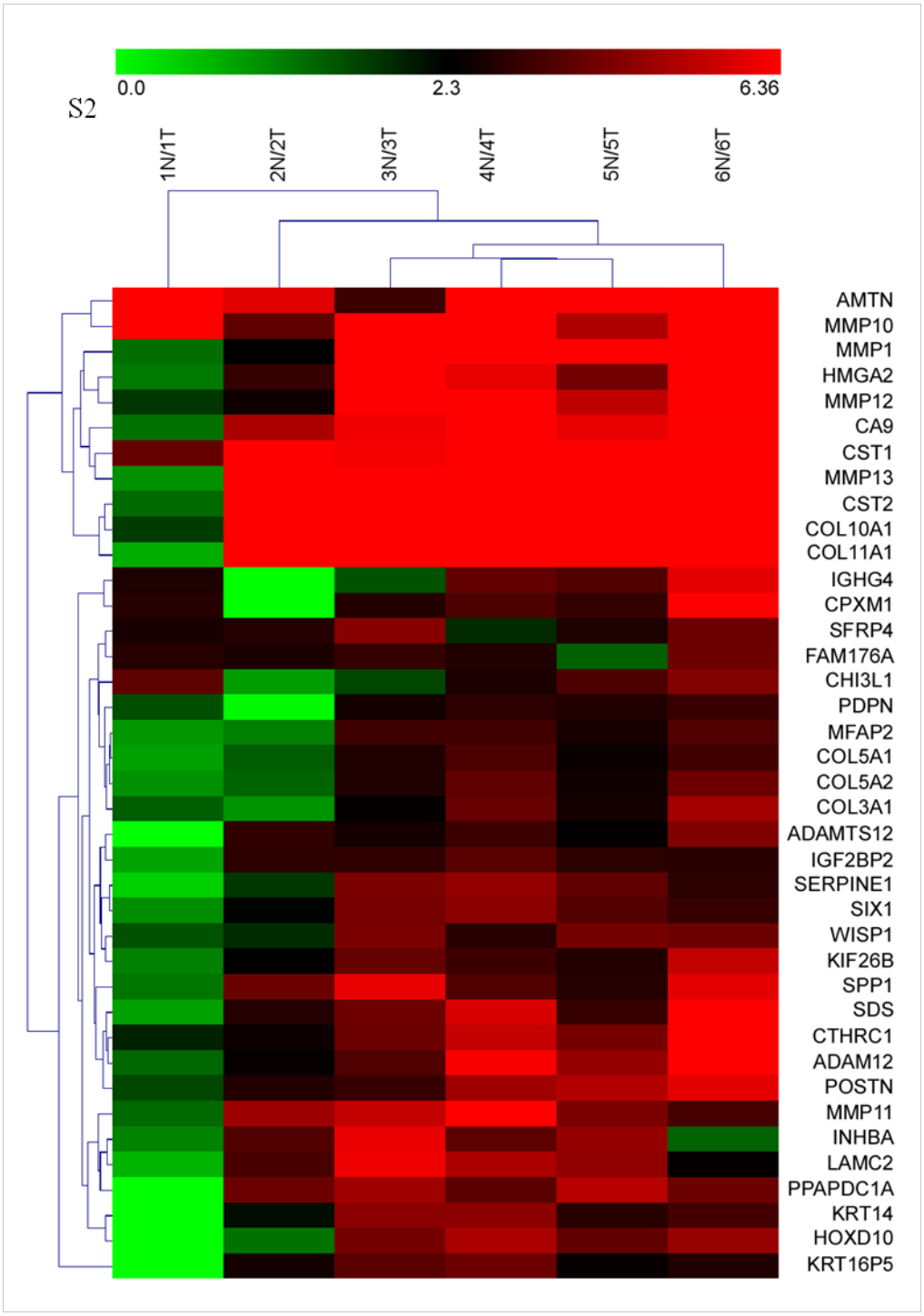
Table S5. The cut-off values for CHI3L1, MMP13, and SPP1 for each stage.

	Case number of ESCC	CHI3L1 (ng/ml)	MMP13 (ng/ml)	SPP1 (ng/ml)
Test cohort				
Stage I	20	25.19	0.62	19.08
Stage II	51	27.15	0.43	19.87
Stage III	71	42.78	0.61	30.74
Stage IV	8	42.29	0.39	29.78
Validation cohort				
Stage I	13	31.37	0.58	17.48
Stage II	71	33.49	0.70	17.08
Stage III	69	34.62	0.41	19.38
Stage IV	16	34.01	0.66	25.55

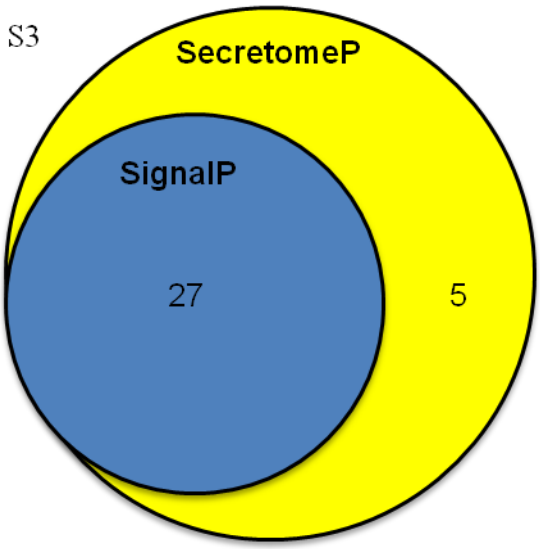
Table S6 Levels of CHI3L1, MMP13 and SPP1 and clinical characteristics of patients with ESCC

Characteristics	case numbers	CHI3L1(ng/ml)		MMP13(ng/ml)		SPP1(ng/ml)	
		Median(range)	*P*	Median(range)	*P*	Median(range)	*P*
Age, years			**0.0002**		**0.0267**		0.224
<60	160	73.03(11.56-360.50)		4.83(0.03-29.50)		78.36(7.04-203.40)	
≥60	159	100.8(8.05-430.80)		4.08(0.01-26.00)		84.25(5.12-233.40)	
Gender			0.5233		0.6963		**0.0037**
Male	237	78.70(11.56-430.80)		4.55(0.01-29.50)		86.68(5.12-233.40)	
Female	82	84.46(8.05-419.20)		4.55(0.15-26.00)		67.78(7.65-225.10)	
pT status			0.598		0.3531		**0.0165**
pT1	39	78.18(15.40-264.70)		3.97(0.01-14.92)		72.81(8.07-187.50)	
pT2	50	92.96(19.20-430.80)		4.55(0.11-17.13)		90.71(8.99-174.60)	
pT3	197	79.23(8.05-421.30)		4.55(0.02-27.41)		77.91(5.12-233.40)	
pT4	33	95.80(27.32-361.00)		5.11(0.16-29.50)		111.10(14.92-200.50)	
pN status			0.3683		0.2471		0.4014
pN0	143	78.70(11.56-430.80)		5.16(0.01-29.50)		83.05(8.90-225.10)	
pN1	125	81.64(8.05-421.30)		4.04(0.16-27.41)		86.40(7.04-228.40)	
pN2	38	85.48(30.08-361.00)		4.65(0.17-16.99)		66.35(11.91-211.40)	
pN3	12	61.62(24.27-185.60)		3.14(0.42-11.97)		68.83(5.12-157.00)	
pM status			0.7975		0.3825		0.6224
pM0	295	81.44(8.05-430.80)		4.55(0.01-29.50)		80.01(5.12-228.40)	
pM1	24	67.76(21.64-419.20)		4.30(0.02-13.45)		88.26(7.04-233.40)	
pTNM status			0.3401		0.6853		0.8656
Stage I	33	80.48(15.40-264.70)		4.36(0.01-14.92)		75.98(9.50-187.50)	
Stage II	122	78.52(11.56-430.80)		4.57(0.03-26.00)		78.32(7.65-225.10)	
Stage III	140	85.00(8.05-421.30)		4.54(0.16-29.50)		83.29(5.12-228.40)	
Stage IV	24	67.76(21.64-419.20)		4.30(0.02-13.45)		88.26(7.04-233.40)	

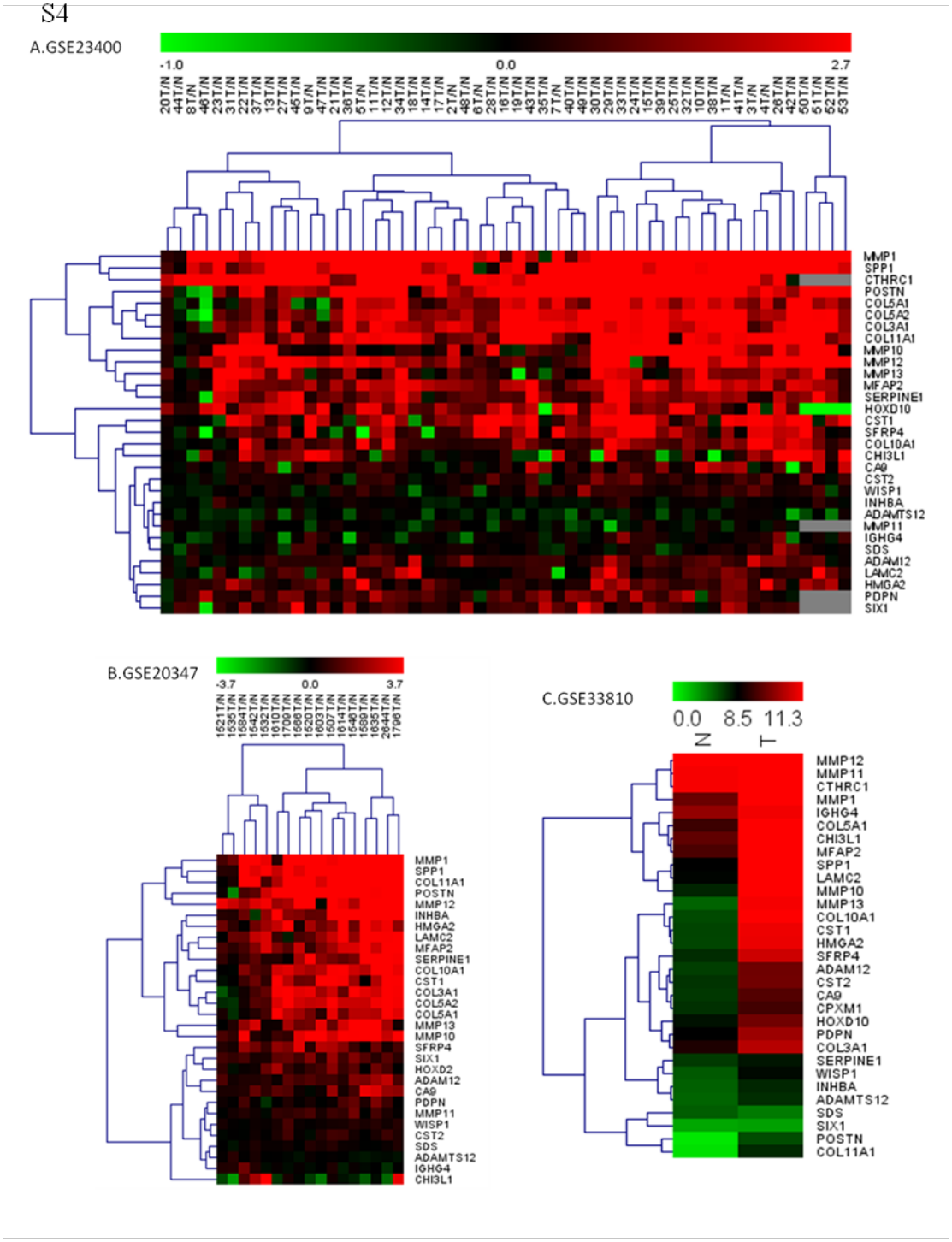

S1
1N/1T
2N/2T
3N/3T
4N/4T
5N/5T
6N/6T


S2
0.0
2.3
6.36
1N/1T
2N/2T
3N/3T
4N/4T
5N/5T
6N/6T
AMTN
MMP10
MMP1
HMGA2
MMP12
CA9
CST1
MMP13
CST2
COL10A1
COL11A1
IGHG4
CPXM1
SFRP4
FAM176A
CHI3L1
PDPN
MFAP2
COL5A1
COL5A2
COL3A1
ADAMTS12
IGF2BP2
SERPINE1
SIX1
WISP1
KIF26B
SPP1
SDS
CTHRC1
ADAM12
POSTN
MMP11
INHBA
LAMC2
PPAPDC1A
KRT14
HOXD10
KRT16P5

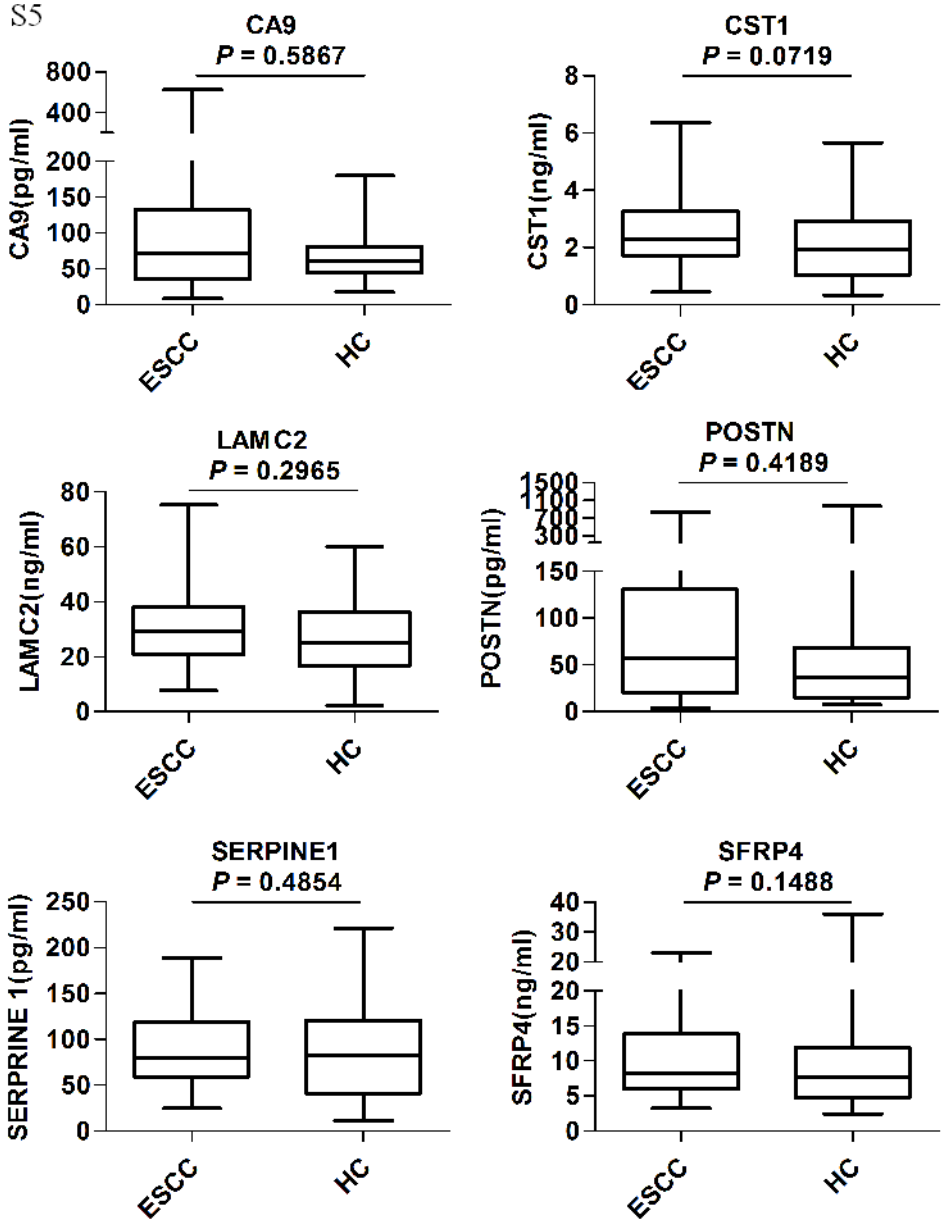
S3



S4

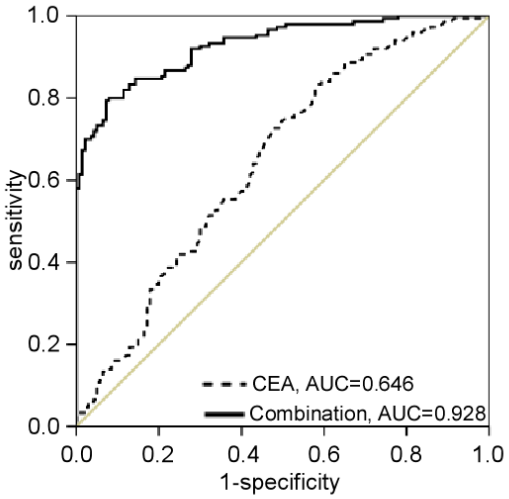

A.GSE23400
-1.0
0.0
2.7
20T/N
44T/N
8T/N
46T/N
23T/N
31T/N
22T/N
37T/N
13T/N
27T/N
45T/N
9T/N
47T/N
21T/N
36T/N
5T/N
11T/N
12T/N
34T/N
18T/N
14T/N
17T/N
2T/N
48T/N
6T/N
28T/N
16T/N
19T/N
43T/N
35T/N
7T/N
40T/N
49T/N
30T/N
29T/N
33T/N
24T/N
15T/N
39T/N
25T/N
32T/N
10T/N
38T/N
1T/N
41T/N
3T/N
4T/N
26T/N
42T/N
50T/N
51T/N
52T/N
53T/N
MMP1
SPP1
CTHRC1
POSTN
COL5A1
COL5A2
COL3A1
COL11A1
MMP10
MMP12
MMP13
MFAP2
SERPINE1
HOXD10
CST1
SFRP4
COL10A1
CHI3L1
CA9
CST2
WISP1
INHBA
ADAMTS12
MMP11
IGHG4
SDS
ADAM12
LAMC2
HMGA2
PDPN
SIX1
B.GSE20347
-3.7
0.0
3.7
1521T/N
1535T/N
1584T/N
1542T/N
1532T/N
1610T/N
1709T/N
1566T/N
1623T/N
1507T/N
1614T/N
1546T/N
1589T/N
1635T/N
2644T/N
1706T/N
MMP1
SPP1
COL11A1
POSTN
MMP12
INHBA
HMGA2
LAMC2
MFAP2
SERPINE1
COL10A1
CST1
COL3A1
COL5A2
COL5A1
MMP13
MMP10
SFRP4
SIX1
HOXD2
ADAM12
CA9
PDPN
MMP11
WISP1
CST2
SDS
ADAMTS12
IGHG4
CHI3L1
C.GSE33810
0.0
8.5
11.3
N
T
MMP12
MMP11
CTHRC1
MMP1
IGHG4
COL5A1
CHI3L1
MFAP2
SPP1
LAMC2
MMP10
MMP13
COL10A1
CST1
HMGA2
SFRP4
ADAM12
CST2
CA9
CPXM1
HOXD10
PDPN
COL3A1
SERPINE1
WISP1
INHBA
ADAMTS12
SDS
SIX1
POSTN
COL11A1

S5

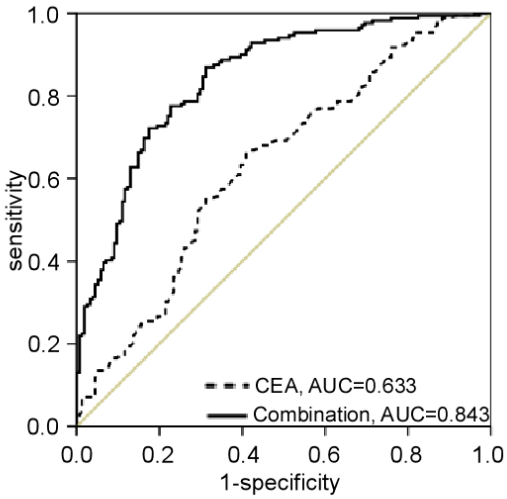

CA9
P = 0.5867
CA9(pg/ml)
800
400
200
150
100
50
0
ESCC
HC
CST1
P = 0.0719
CST1(ng/ml)
8
6
4
2
0
ESCC
HC
LAMC2
P = 0.2965
LAMC2(ng/ml)
80
60
40
20
0
ESCC
HC
POSTN
P = 0.4189
POSTN(pg/ml)
1500
1100
700
300
150
100
50
0
ESCC
HC
SERPINE1
P = 0.4854
SERPRINE 1(pg/ml)
250
200
150
100
50
0
ESCC
HC
SFRP4
P = 0.1488
SFRP4(ng/ml)
40
30
20
20
15
10
5
0
ESCC
HC

S6

A Test Cohort



B Validation Cohort



C

Marker(ng/ml)	AUC	Cut-off	Sen(%)	Spe(%)	PPV(%)	NPV(%)
Test-CEA	0.646	5.0	6.7	95.7	62.5	48.91
Validation-CEA	0.633	5.0	13.0	94.8	73.33	49.83