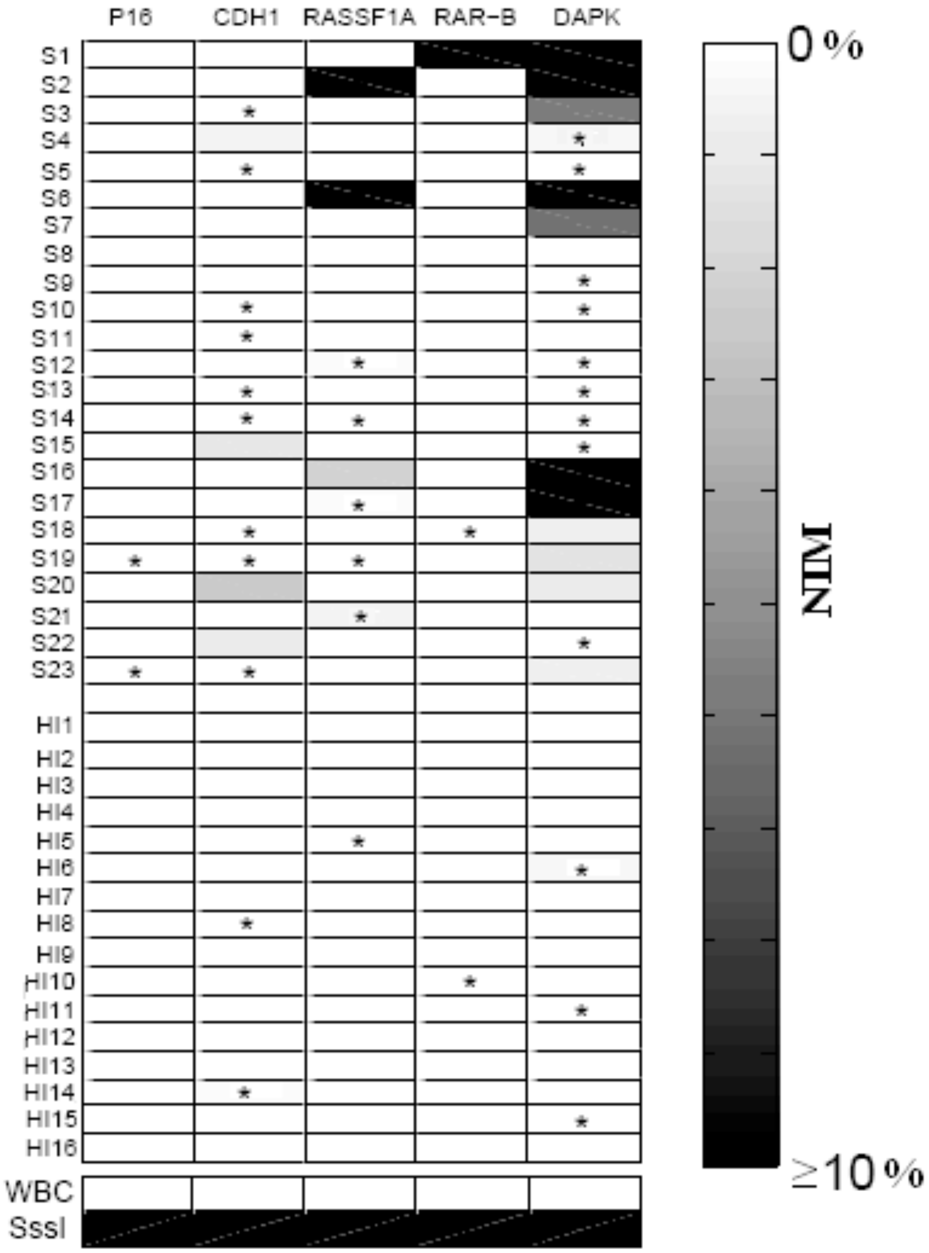
Supplementary figure 1.



Supplementary table 1. List of primer and probe sequences

Genes	primer sequences	Annealing temperature (°C)	Product size (bp)
(A) primer sequences for MSP			
p16	M: sense: TTATTAGAGGGTGGGGCGGATCGC[a]	65	150
	antisense: GACCCCGAACCGCGACCGTAA		
	U: sense: TTATTAGAGGGTGGGGTGGATTGT	60	151
	antisense: CAACCCCAAACCACAACCATAA		
p14	M: sense：GTGTTAAAGGGCGGCGTAGC	60	122
	antisense：AAAACCCTCACTCGCGACGA		
	U： sense：TTTTTGGTGTTAAAGGGTGGTGTAGT	60	132
	antisense：CACAAAAACCCTCACTCACAACAA		
APC	M: sense: TATTGCGGAGTGCGGGTC	55	98
	antisense: TCGACGAACTCCCGACGA		
	U: sense:GTGTTTTATTGTGGAGTGTGGGTT	60	108
	antisense:CCAATCAACAAACTCCCAACAA		
MGMT	M: sense: TTTCGACGTTCGTAGGTTTTCGC	59	81
	antisense: GCACTCTTCCGAAAACGAAACG		
	U: sense: TTTGTGTTTTGATGTTTGTAGGTTTTTGT	59	93
	antisense: AACTCCACACTCTTCCAAAAACAAAACA		
DAPK	M: sense: GGATAGTCGGATCGAGTTAACGTC	60	98
	antisense: CCCTCCCAAACGCCGA		
	U: sense: GGAGGATAGTTGGATTGAGTTAATGTT	60	106
	antisense: CAAATCCCTCCCAAACACCAA		
RASSF1A	M: sense: GTGTTAACGCGTTGCGTATC	60	94
	antisense: AACCCCGCGAACTAAAAACGA		
	U: sense: TTTGGTTGGAGTGTGTTAATGTG	60	108
	antisense: CAAACCCCACAAACTAAAAACAA		
CDH1	M: sense: TTAGGTTAGAGGGTTATCGCGT	57	115
	antisense: TAACTAAAAATTCACCTACCGAC		

	U: sense: TAATTTTAGGTTAGAGGGTTATTGT	53	97
	antisense: CACAACCAATCAACAACACA		
ADAM23	M: sense: GGGCGTACGTTCGTTTC	62	117
	antisense: CAACGACTACGAAAACTACCG		
	U: sense: GGGGTGGGGTGTATGTTT	62	135
	antisense: ACACAACCACTTCAACAACTACA		
RAR-β	M: sense: TCGAGAACGCGAGCGATTCG	60	146
	antisense: GACCAATCCAACCGAAACGA		
	U: sense: TTGAGAATGTGAGTGATTTGA	60	146
	antisense: AACCAATCCAACCAAAACAA		
TIMP-3	M: sense: CGTTTCGTTATTTTTTGTTTTCGGTTTC	59	116
	antisense: CCGAAAACCCCGCCTCG		
	U：sense：TTTTGTTTTGTTATTTTTTGTTTTTGGTTTT	59	122
	antisense：CCCCCAAAAACCCCACCTCA		
GSTP1	M: sense：TTCGGGGTGTAGCGGTCGTC	59	91
	antisense：GCCCCAATACTAAATCACGACG		
	U： sense：GATGTTTGGGGTGTAGTGGTTGTT	59	97
	antisense：CCACCCCAATACTAAATCACAACA		
FHIT	M: sense:TTGGGGCGCGGGTTTGGGTTTTTACGC	66	74
	antisense:CGTAAACGACGCCGACCCCACTA		
	U: sense:TTGGGGTGTGGGTTTGGGTTTTTATG	66	74
	antisense:CATAAACAACACCAACCCCACTA		
(B) primer sequences for qRT-PCR			
ACTB	sense: TCGTGCGTGACATTAAGGAG	65	106
	antisense: GGCAGCTCGTAGCTCTTCTC		
DNMT1	sense: TACCTGGACGACCCTGACCTC	65	103
	antisense: CGTTGGCATCAAAGATGGACA		
DNMT3a	sense: TATTGATGAGCGCACAAGAGAGC	65	111

	antisense: GGGTGTTCCAGGGTAACATTGAG		
DNMT3b	sense: GGCAAGTTCTCCGAGGTCTCTG	65	113
	antisense: TGGTACATGGCTTTTCGATAGGA		
(C) primer and probe sequences for MethyLight			
ACTB	sense: TGGTGATGGAGGAGGTTTAGTAAGT	60	133
	antisense: AACCAATAAAACCTACTCCTCCCTTAA		
	probe: FAM-ACCACCACCCAACACACAATAACAAACACA-TAMRA		
DAPK	sense: GGATAGTCGGATCGAGTTAACGTC	60	98
	antisense: CCCTCCCAAACGCCGA		
	probe: FAM-ACCCTACCGCTACGAATTACCGAATCCCCT-TAMRA		
p16	sense: TGGAGTTTTCGGTTGATTGGTT	60	70
	antisense: AACAACGCCCGCACCTCCT		
	probe: FAM-ACCCGACCCCGAACCGCG-TAMRA		
RASSF1A	sense: GCGTTGAAGTCGGGGTTC	60	75
	antisense: CCCGTACTTCGCTAACTTTAAACG		
	probe: FAM-ACAAACGCGAACCGAACGAAACCA-TAMRA		
RAR-β	sense: GGGATTAGAATTTTTTATGCGAGTTGTTGT	60	93
	antisense: TACCCCGACGATACCCAAAC		
	probe: FAM-CGAATCGCTCGCGTTCTCGACA-TAMRA		
CDH1	sense: AATTTTAGGTTAGAGGGTTATCGCGT	60	70
	antisense: TCCCCAAAACGAAACTAACGAC		
	probe: FAM-CGCCCACCCGACCTCGCAT-TAMRA		

qRT-PCR, quantitative reverse transcription-PCR; M, methylated- specific primers; U, unmethylated-specific primers.

Supplementary table 2. Correlation between serum DNA hypermethylation and clinico-pathological parameters of ESCC patients (n=45)

	Frequency of methylation (%)					
	p16	RASSF1A	DAPK	RAR-β	CDH1	≥2 genes
Gender						
Male	8.3	63.9	72.2	19.4	83.3	80.6
Female	0.0	55.6	77.8	55.6	88.9	88.9
P-value	1	0.711	1	0.043	1	1
Age group (year)						
＜60	12.5	75.0	75.0	29.2	87.5	87.5
≥60	0.0	47.6	71.4	23.8	81.0	76.2
P-value	0.236	0.073	1	0.746	0.689	0.443
Size (cm)*						
＜5	4.3	65.2	65.2	34.8	78.3	78.3
≥5	9.5	57.1	85.7	19.0	90.5	85.7
P-value	0.599	0.758	0.169	0.318	0.416	0.701
Location of tumor*						
Upper	0.0	40.0	100.0	40.0	80.0	80.0
Middle	8.3	62.5	66.7	29.2	79.2	79.2
Lower	6.7	66.7	80.0	20.0	93.3	86.7
P-value	1	0.617	0.327	0.626	0.492	0.864
Differentiation *						
Poorly	11.8	58.8	70.6	41.2	88.2	82.4
Moderately	5.0	60.0	85.0	20.0	80.0	80.0
Well	0.0	71.4	57.1	14.3	85.7	85.7
P-value	0.756	0.844	0.331	0.341	0.858	1
pT category *						
1-2	9.1	63.6	45.5	27.3	63.6	54.5
3-4	6.1	60.6	84.8	27.3	90.9	90.9
P-value	1	1	0.016	1	0.054	0.016
pN category *						
0	9.1	54.5	68.2	22.7	86.4	81.8
1-2	4.5	68.2	81.8	31.8	81.8	81.8
P-value	1	0.537	0.488	0.736	1	1
Smoking*						
Smoker	9.4	62.5	75.0	21.9	81.3	78.1
Non-smoker	0.0	58.3	75.0	41.7	91.7	91.7
P-value	0.551	1	1	0.259	0.653	0.413
Alcohol *						
Drinker	10.7	60.7	82.1	28.6	89.3	82.1

Non-drinker	0.0	62.5	62.5	25.0	75.0	81.3
P-value	0.29	1	0.169	1	0.236	1

*, Clinical data was not available for one patient.

Supplementary Table 3. Diagnostic information of serum DNA methylation at 5 genes in another serum sample validation set

Gene	*AUC (95%, CI)*	*Sensitivity (%)*	**Specificity (%)**
p16	0.544(0.485-0.602)	8.7	100
DAPK	0.841(0.720-0.962)	87.0	81.3
RAR-β	0.512(0.427-0.597)	8.7	93.8
CDH1	0.720(0.587-0.853)	56.5	87.5
RASSF1A	0.643(0.526-0.760)	34.8	93.8
Any gene	0.760(0.627-0.892)	95.7	56.3
2 or more genes	0.891(0.805-0.978)	78.3	100.0

CI, confidence interval.