

## **Supplementary Material for**

# **Elafin as a Prognostic Marker in Esophageal Squamous Cell Carcinoma: A Pilot Study using 3D Imaging and Genomic Profiling**

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**This supplementary material includes:**

**Description of dataset 1**

**Table S1 to Table S5**

**Figure S1 to Figure S5**

**Video S1 to Video S4**

**References of Table S5**

Dataset 1: Whole gene expression of CE81T2-myc (overexpression control), CE81T2-elafin-myc (elafin overexpression), CE81T2-4 scramble control (knock down control), and CE81T2-4-Sh6 (elafin-knock down) by next generation sequencing service of Agilent Technologies.

This database already uploaded on Gene Expression Omnibus database as GSE205623, and will be available to the public once this article is accepted.

**Table S1. (A) Number of layers and mean scores of elafin intensity in each cluster of the two comparison pairs. (B) Ranking of the observed mean difference (in red) of the clustered elafin intensity scores among all random reallocations.**

(A)

Cluster	Pair 1				Pair 2			
	E365		E385		E535		E421	
	N (% of 1)	Mean						
1	9 (100)	1	9 (0)	0	9 (0)	0	10 (0)	0
2	10 (100)	1	9 (0)	0	10 (50)	0.5	11 (0)	0
3	10 (100)	1	9 (0)	0	10 (100)	1	11 (0)	0
4	10 (20)	0.2	9 (0)	0	9 (100)	1	11 (0)	0
5	10 (0)	0	9 (0)	0	10 (100)	1	10 (0)	0
6	10 (0)	0	10 (0)	0	10 (40)	0.4	11 (0)	0
7	10 (0)	0	9 (0)	0	9 (0)	0	11 (0)	0
8	10 (0)	0	9 (0)	0	10 (0)	0	11 (0)	0
9	10 (0)	0	9 (0)	0	10 (0)	0	11 (0)	0
10	10 (0)	0	9 (0)	0	9 (0)	0	10 (0)	0
11	10 (0)	0	9 (0)	0	10 (0)	0	11 (0)	0
12	10 (0)	0	9 (0)	0	10 (0)	0	11 (0)	0
13	10 (0)	0	10 (0)	0	10 (0)	0	11 (0)	0
14	10 (0)	0	9 (0)	0	9 (0)	0	11 (0)	0
15	10 (0)	0	9 (0)	0	10 (0)	0	10 (0)	0
16	10 (0)	0	9 (0)	0	10 (0)	0	11 (0)	0
17	10 (0)	0	9 (0)	0	9 (0)	0	11 (0)	0
18	10 (0)	0	9 (0)	0	10 (0)	0	11 (0)	0
19	10 (0)	0	9 (0)	0	10 (0)	0	11 (0)	0
20	10 (0)	0	9 (0)	0	9 (0)	0	10 (0)	0

(B)

Pair 1 (E365 vs. E385)		Pair 2 (E535 vs. E421)			
Mean difference	Frequency	Mean difference	Frequency	Mean difference	Frequency
-0.16	65536	-0.195	32768	0.005	98304
-0.14	65536	-0.155	32768	0.045	98304
-0.06	196608	-0.145	32768	0.055	98304
-0.04	196608	-0.105	32768	0.095	98304
0.04	196608	-0.095	98304	0.105	32768
0.06	196608	-0.055	98304	0.145	32768
0.14	65536	-0.045	98304	0.155	32768
<b>0.16</b>	<b>65536</b>	-0.005	98304	<b>0.195</b>	<b>32768</b>

**Table S2. Baseline characteristics of 63 locoregional ESCC patients for tissue immunohistochemistry staining of elafin.**

Elafin protein expression in tissue arrays (n = 63) <sup>a</sup>		P value
Male (%)	62 (98.4)	
Age, yrs	57.17±11.62	
Tumor score	0	5 (7.9)
	1	22 (34.9)
	1.5	1 (1.6)
	2	14 (22.2)
	3	16 (25.4)
	missing	5 (7.9)
Normal score	0	3 (4.8)
	1	26 (41.3)
	1.5	4 (6.3)
	2	8 (12.7)
	missing	22 (34.9)
<b>Mean score (± SD)</b>		
Normal only (n = 41)	1.17±0.52	
Tumor only (n = 58)	1.72±0.97	<b>&lt;0.0001<sup>b</sup></b>
Difference in paired (T-N) (n = 36)	0.53±1.10	<b>0.006<sup>c</sup></b>

<sup>a</sup>Data are presented as n (%) or mean ± SD.

<sup>b</sup>Wilcoxon rank sum test.

<sup>c</sup>Wilcoxon signed ranked test.

**Table S3. Elafin promoted the proliferation of ESCC cell lines.**

Elafin manipulation	Doubling time (hours)			
	CE81T2	KYSE270	OE21	CE81T2-4
Overexpression				
Myc	24.6±1.5	29.4±1.3	NA	NA
Elafin-myc	20.7±0.7 <sup>a</sup>	25.7±0.9 <sup>a</sup>	NA	NA
Knockdown				
Scramble	NA	NA	20.5±0.4	21.4±0.3
shElafin #1	NA	NA	26.5±1.4 <sup>b</sup>	26.3±0.2 <sup>b</sup>
shElafin #6	NA	NA	26.5±2.4 <sup>b</sup>	25.6±0.02 <sup>b</sup>

NA. not available

<sup>a</sup> $P < 0.05$ , compared to myc control cells

<sup>b</sup> $P < 0.05$ , compared to the scramble control cells

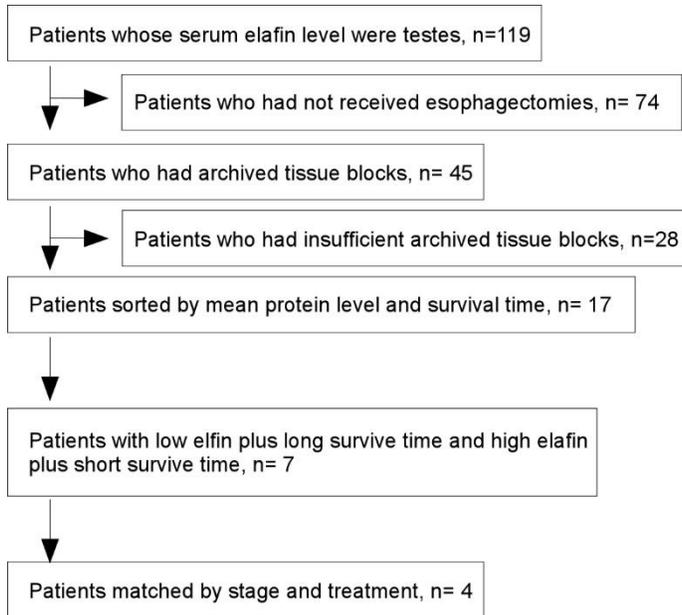
**Table S4. Gene expression that are related with mesenchymal and epithelial markers after overexpressing in CE81T2 or knockdowning in CE81T2-4.**

	Gene symbol	CE81T2_Elafin/ CE81T2_myc (FPKM Log <sub>2</sub> ratio)	CE81T2-4_Sh6/ CE81T2-4_Scr (FPKM Log <sub>2</sub> ratio)
Epithelial markers	FOXA2	0.429599989	0.64291716
	MST1R	0.151935118	0.250063583
	CTNNA1	0.020569151	0.210860369
	PTP4A1	0.022118376	0.132822609
	GSC	0	10.87502744
	KRT32	0	1.137443009
	CDH1	-0.028653927	0.084503857
	LAMA3	-0.025543071	0.062583531
	CTNNA1	-0.128441712	0.352366815
	KRT7	-0.127838992	0.037778945
Mesenchymal markers	FGFBP1	-0.375034632	-0.036013612
	ZEB1	2.818437436	-2.658923843
	NES	0.673191947	-0.742560707
	SPP1	0.44743237	-1.651540783
	SOX9	0.411527454	0.380251678
	CTGF	0.388643493	0.178817974
	TWIST1	0.367803664	0.012048495
	MMP3	0.364003753	-15.21787442
	TMEM132A	0.343728724	0.049315448
	MMP2	0.336531555	0.188936685
	GNG11	0.325871007	-0.704125902
	IGFBP4	0.279090455	-0.606191217
	AHNAK	0.287340771	-0.32698529
	EGFR	0.227689682	-0.005464374
	SPARC	0.205713921	-0.241125458
	CD59	0.20868233	0.03641868
	FN1	0.161171404	-0.946917859
	FOXF1	0.15501339	-0.1450207
	GATA6	0.097544498	-0.498625639
	ZNF703	0.073572496	0.041974501
ITGAV	0.0634668	-0.064261071	
NUDT13	-0.110979642	-0.326959105	
FGFR2	-0.134549924	-0.292310771	

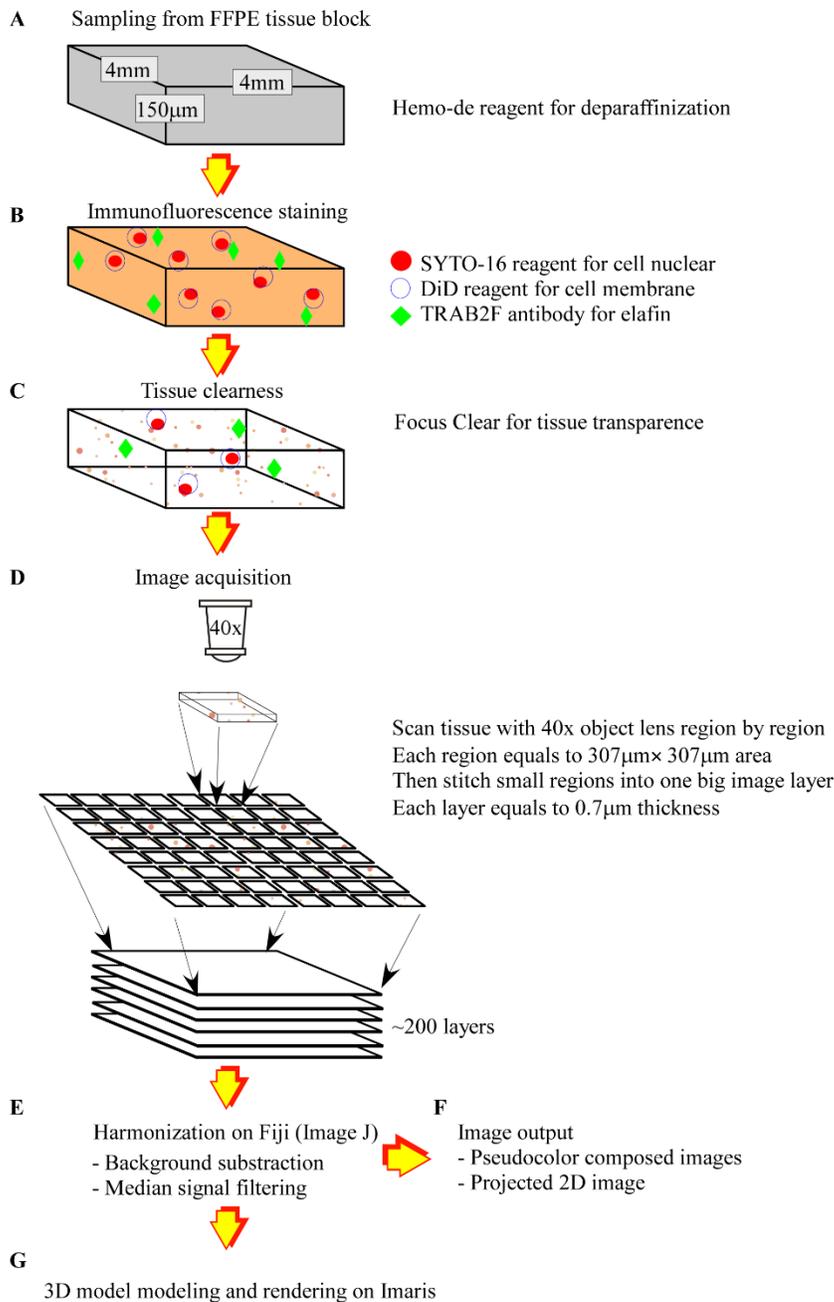
Abbreviation: FPKM= Fragments Per Kilobase of transcript per Million

**Table S5. Summary of elafin expressions in different human cancers.**

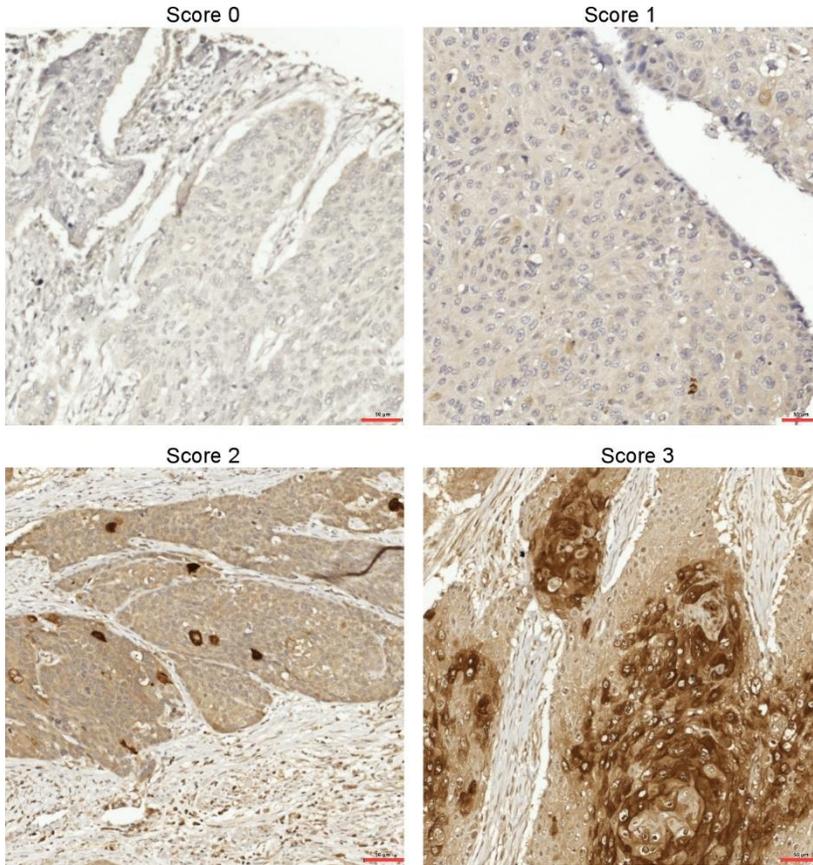
Cancer type	Level (RNA or Protein)	Up or down regulated (Cancer tissue vs. normal tissue)	References
<b>Arise from squamous epithelium</b>			
Esophageal cancer	Protein	Up	(1)
Head and neck cancer	Protein	Up	(2)
Lung cancer	Protein	Up	(3)
<b>Arise from glandular cell</b>			
Breast cancer	Protein	Up	(4)
	RNA	Up	(5)
	RNA & Protein	Down	(6)
	RNA & Protein	Down	(7)
	RNA & Protein	Down	(8)
	Protein	Down	(9)
Ovarian cancer	RNA & Protein	Up	(10)
	RNA & Protein	Not available	(11)
	RNA & Protein	Up	(12)
Colorectal cancer	RNA & Protein	Not available	(13)
<b>Others</b>			
Glioblastoma	RNA & Protein	Up	(14)
	RNA & Protein	Up	(15)
Melanoma	RNA & Protein	Down	(16)



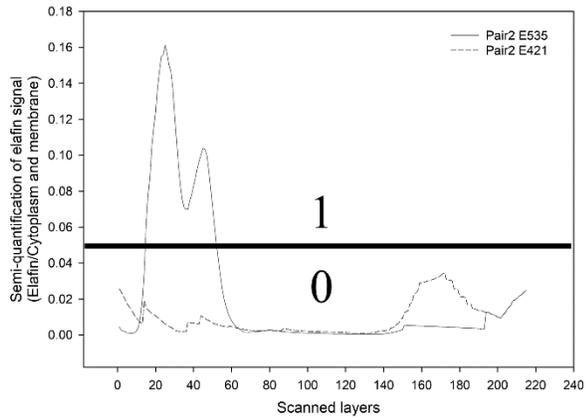
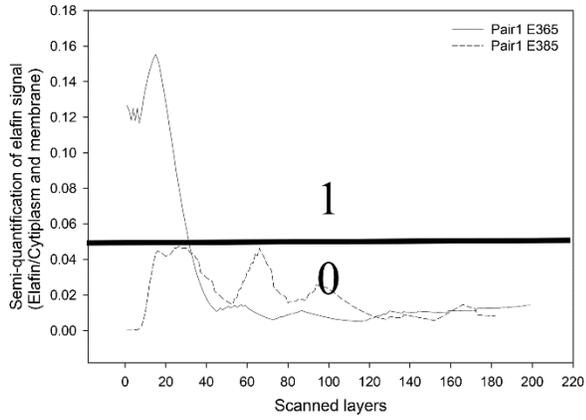
**Figure S1. Scheme of patient selection from the 119 ESCC patients.**



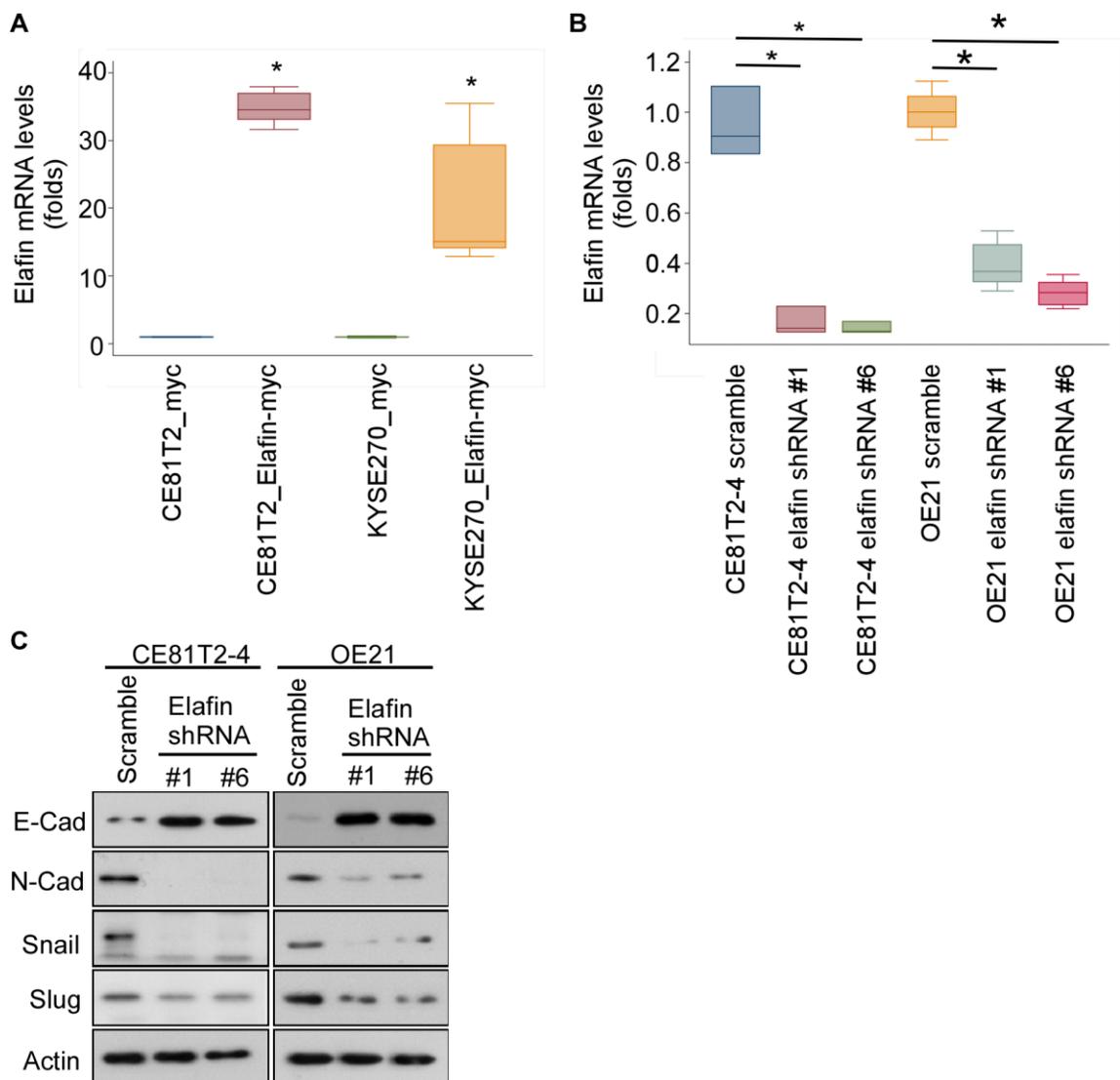
**Figure S2. Flowchart of 3D tissue image acquisition, processing and modeling.** (A) Matched the HE staining slide with FFPE tissue block and sampled tissue region with biopsy needle. (B) Stained tissue chunk with anti-elafin antibody and two reagents (nuclear and cytoplasm). (C) Tissue cleared by FocusClear solution. (D) Images captured with 40x object lens by confocal microscopy and stitched into one image. The whole tissue specimen was optical sectioned into ~200 layers of z-axis. (E) Image harmonized to reduce the noise signal, improve shape of targeted objects, and determine threshold for the construction of 3D architecture. (F) Projected pseudo color image of 200 layers into one layer by Fiji software. (G) Constructed and rendered 3D model of elafin signal and cell nuclear by Imaris software. Abbreviation: FFPE= formalin-fixed, paraffin-embedded



**Figure S3. The scoring of elafin staining signal.** Score 0 = negative cytoplasmic staining. Score 1 = focal weak cytoplasmic staining. Score 2 = diffuse moderate cytoplasmic staining and blurred membrane staining. Score 3 = diffuse strong cytoplasmic stain and distinct membrane staining. Immunohistochemistry was performed by elafin antibody on a tissue microarray. The representative images show the scoring of elafin signal mentioned in Materials and Methods. Scale bar on bottom right=50 $\mu$ m.



**Figure S4. Dichotomous score of the elafin intensities of the tumor vs. normal tissues of the two comparison pairs.**



**Figure S5. Elafin mRNA levels after transfecting with elafin-expression vector or elafin shRNAs in cell lines with different endogenous elafin levels.** (a) Elafin mRNA expression levels increased in the CE81T2 and KYSE270 cell lines after transfecting with ectopic elafin. (b) Elafin mRNA levels decreased in the CE81T2-4 and OE21 cells after being treated with elafin shRNAs. \*P<0.05, compared to the controls. (c) Knockdown elafin by elafin-specific shRNAs altered EMT markers in CE81T2-4 and OE21 cell lines

## Supplementary Videos

**Video S1. The 3-D cuboid of yellow square in figure 2A (E365).** The pseudo color green is for elafin, blue for cell membrane and cytoplasm, and red for cell nuclei. Scale bar = 30  $\mu\text{m}$

**Video S2. The 3-D cuboid of yellow square in figure 2G (E535).** The pseudo color green is for elafin, blue for cell membrane and cytoplasm, and red for cell nuclei. Scale bar = 30  $\mu\text{m}$

**Video S3. The 3-D cuboid of yellow square in figure 2D (E385).** The pseudo color green is for elafin, blue for cell membrane and cytoplasm, and red for cell nuclei. Scale bar = 30  $\mu\text{m}$

**Video S4. The 3-D cuboid of yellow square in figure 2J (E421).** The pseudo color green is for elafin, blue for cell membrane and cytoplasm, and red for cell nuclei. Scale bar = 30  $\mu\text{m}$

## Supplementary References

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