Inhibition of 6-phosphogluconate dehydrogenase suppresses esophageal squamous cell carcinoma growth and enhances the anti-tumor effects of metformin via the AMPK/mTOR pathway

Bei Wang[1,2,3†], Zixuan Wang[5,6†], Zini Zhou[4,5], Gui Liu[4,7], Zhenyuan Jiang[5,6], Mingyue Zheng[2,3], Wei Geng[1,4,5,7*]

1.Yancheng Key Laboratory of Molecular Epigenetics, The First people's Hospital of Yancheng, The Yancheng Clinical College of Xuzhou Medical University; 66 South People's Road, Jiangsu, Yancheng 224000, China

2.School of Chinese Materia Medica, Nanjing University of Chinese Medicine; 138 Xianlin Road, Jiangsu, Nanjing 210023, China

3.Drug Discovery and Design Center, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences; 555 Zuchongzhi Road, Shanghai 201203, China

4.Department of Radiotherapy, The First people's Hospital of Yancheng, The Yancheng Clinical College of Xuzhou Medical University; 66 South People's Road, Jiangsu, Yancheng 224000, China

5.Xuzhou Medical University; 209 Tongshan Road, Xuzhou 221004, China

6.Department of Radiotherapy, Jiangsu Cancer Hospital, The Affiliated Cancer Hospital of Nanjing Medical University; 42 Baizi Pavilion, Jiangsu, Nanjing 210009, China

7.School of Medicine, Jiangsu University; 301 Xuefu Road, Jiangsu, Zhenjiang 212013, China

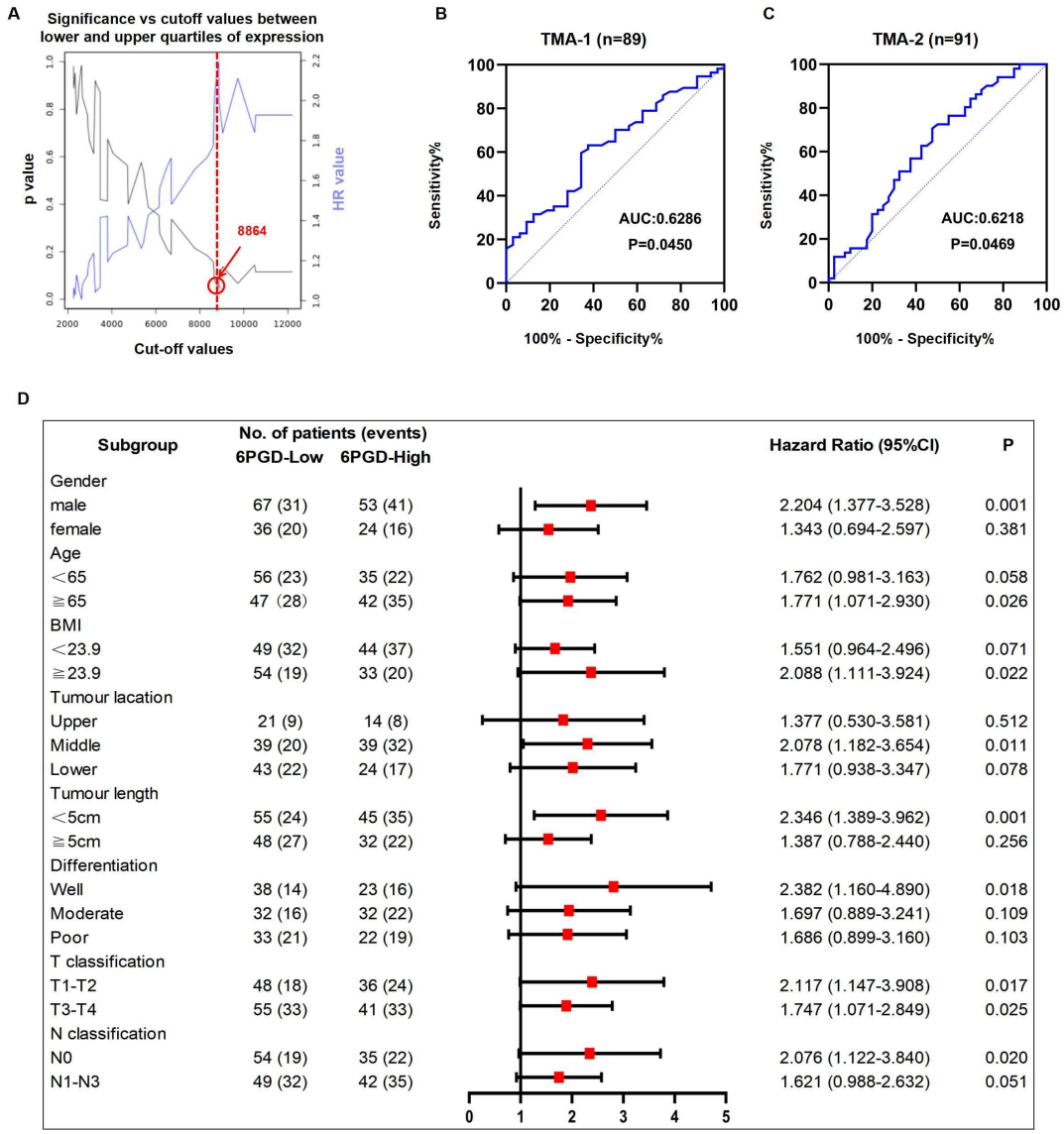
† These authors contributed equally to this work.

*Corresponding author. Email: weihuo2001@163.com (Wei Geng).

This PDF file includes:

Figures S1 to S8

Tables S1 & S2



Subgroup	No. of patients (events) 6PGD-Low	6PGD-High	Hazard Ratio (95%CI)	P
Gender				
male	67 (31)	53 (41)	2.204 (1.377-3.528)	0.001
female	36 (20)	24 (16)	1.343 (0.694-2.597)	0.381
Age				
＜65	56 (23)	35 (22)	1.762 (0.981-3.163)	0.058
≧65	47 (28)	42 (35)	1.771 (1.071-2.930)	0.026
BMI				
＜23.9	49 (32)	44 (37)	1.551 (0.964-2.496)	0.071
≧23.9	54 (19)	33 (20)	2.088 (1.111-3.924)	0.022
Tumour lacation				
Upper	21 (9)	14 (8)	1.377 (0.530-3.581)	0.512
Middle	39 (20)	39 (32)	2.078 (1.182-3.654)	0.011
Lower	43 (22)	24 (17)	1.771 (0.938-3.347)	0.078
Tumour length				
＜5cm	55 (24)	45 (35)	2.346 (1.389-3.962)	0.001
≧5cm	48 (27)	32 (22)	1.387 (0.788-2.440)	0.256
Differentiation				
Well	38 (14)	23 (16)	2.382 (1.160-4.890)	0.018
Moderate	32 (16)	32 (22)	1.697 (0.889-3.241)	0.109
Poor	33 (21)	22 (19)	1.686 (0.899-3.160)	0.103
T classification				
T1-T2	48 (18)	36 (24)	2.117 (1.147-3.908)	0.017
T3-T4	55 (33)	41 (33)	1.747 (1.071-2.849)	0.025
N classification				
N0	54 (19)	35 (22)	2.076 (1.122-3.840)	0.020
N1-N3	49 (32)	42 (35)	1.621 (0.988-2.632)	0.051

Figure S1. The expression level of 6PGD in tumor tissues predicts patient prognosis in ESCC. **(A)** The optimal cut-off value of 6PGD mRNA expression was determined at the peak hazard ratio (HR) and the minimal p-value in the TCGA database. **(B, C)** The cut-off values for 6PGD protein expression in TMA1 (n=89) and TMA2 (n=91) were established using time-dependent receiver operating characteristic (ROC) analysis. **(D)** Forest plot illustrating overall survival (OS) stratified by 6PGD protein levels in different subgroups.

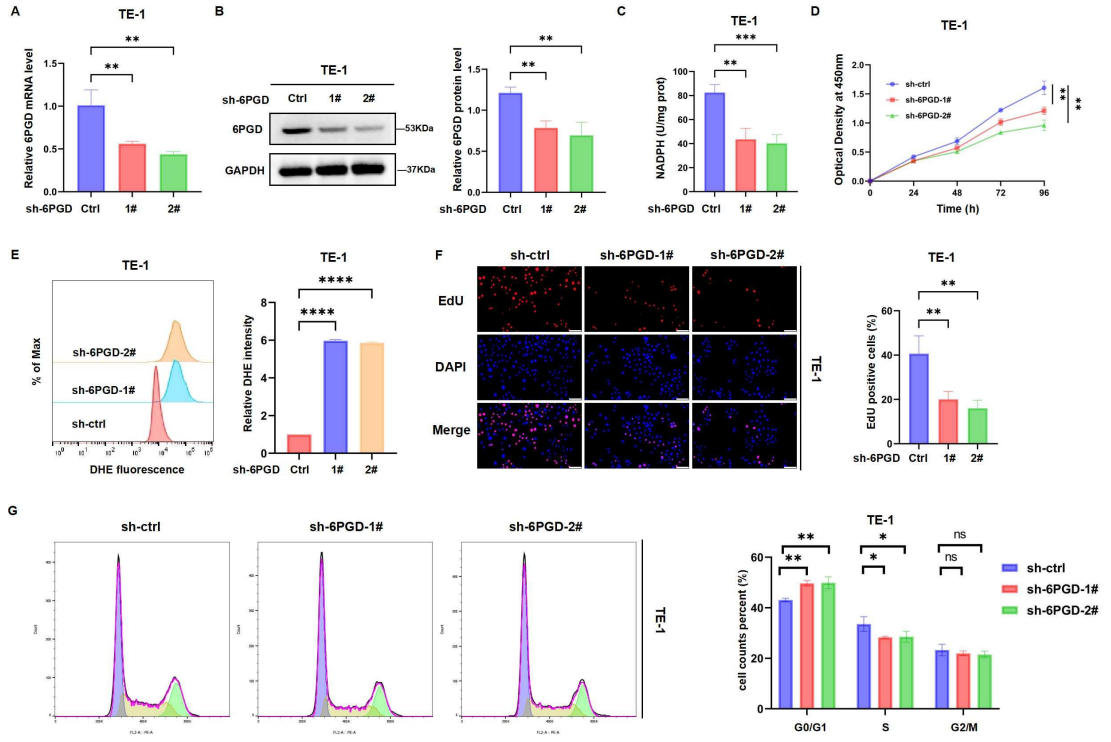


Figure S2. Knockdown of 6PGD suppresses the proliferation of TE-1 cells. (A, B) The knockdown efficiency of 6PGD in TE-1 cells was validated through RT-qPCR and western blot analyses (n=3, mean ± SD). **(C)** The production of NADPH decreased after knocking down 6PGD (n=3, mean ± SD). **(D)** CCK-8 assay was employed to assess the impact of 6PGD knockdown on the viability of TE-1 cells (n=3, mean ± SD). **(E)** The knockdown of 6PGD in TE-1 cells resulted in an elevation of intracellular ROS levels (n=3, mean ± SD). **(F)** The DNA synthesis capacity in TE-1 cells following knockdown of 6PGD was assessed using EdU staining assays (n=3, mean ± SD); Scale bar: 100 μm. **(G)** The alterations in the cell cycle distributions of TE-1 cells following the knockdown of 6PGD (n=3, mean ± SD).

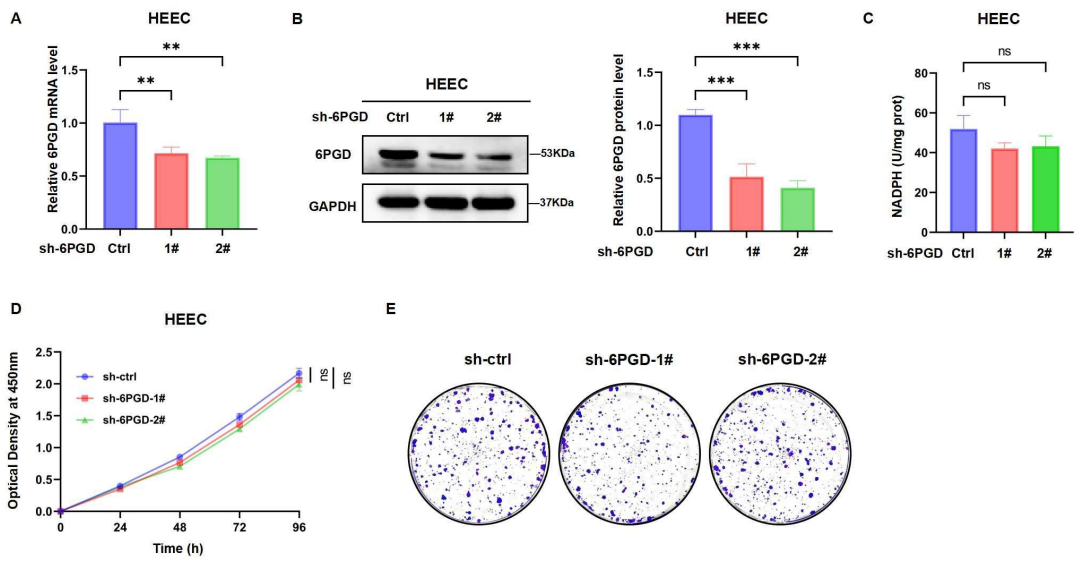


Figure S3. Impact of 6PGD knockdown on the proliferation of normal esophageal epithelial cells. The knockdown efficiency of 6PGD in HEEC cells was validated through RT-qPCR **(A)** and western blot analyses **(B)** (n=3, mean ± SD). The knockdown of 6PGD in HEEC cells had no significant impact on NADPH production **(C)**, cell viability **(D)** or colony-forming capability **(E)**.

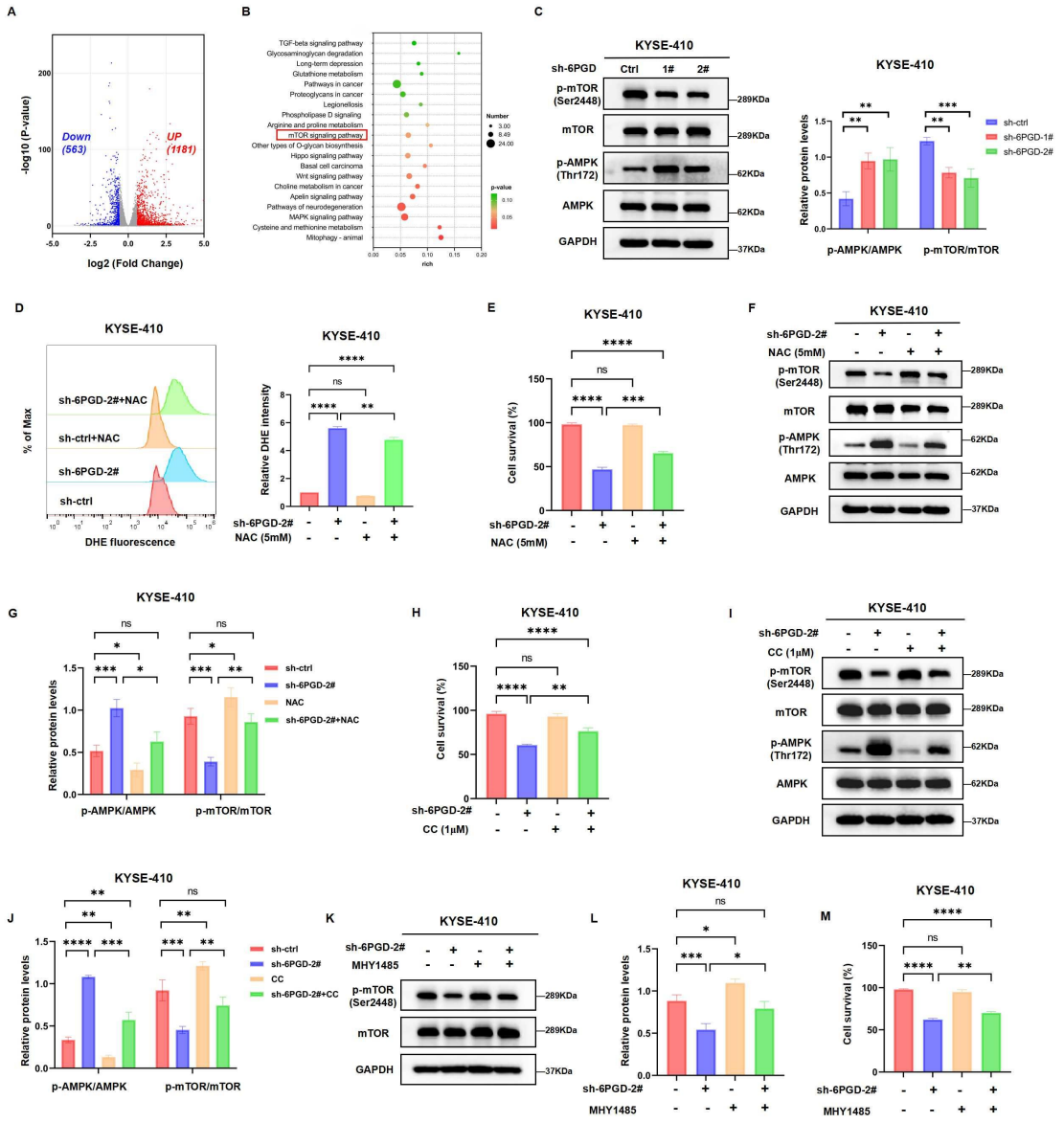


Figure S4. Impact of 6PGD on the AMPK/mTOR signaling pathway in KYSE-410 cells. (A) The volcano plot shows changes in gene expression after 6PGD knockdown in KYSE-410 cells. **(B)** KEGG pathway analysis was performed on the down-regulated genes upon knockdown of 6PGD in KYSE-410 cells. **(C)** Western blot analysis showed increased p-AMPK and decreased p-mTOR levels after 6PGD knockdown in KYSE-410 cells (n=3, mean ± SD). **(D)** The elevation in intracellular ROS levels induced by 6PGD knockdown was reversed by NAC treatment (n=3, mean ± SD). **(E)** NAC mitigates the reduction in cell viability caused by 6PGD knockdown (n=3, mean ± SD). **(F, G)** Western blot analysis showed that NAC treatment effectively reversed the phosphorylation levels of AMPK and mTOR (n=3, mean ± SD). **(H)** The AMPK inhibitor (Compound C) mitigates the reduction in cell viability caused by 6PGD knockdown (n=3, mean ±

SD). **(I, J)** Compound C reversed the increased phosphorylation of AMPK caused by 6PGD knockdown, and subsequently restored the phosphorylation levels of mTOR (n=3, mean ± SD). **(K, L)** The mTOR agonist MHY1485 mitigated the reduction in mTOR phosphorylation induced by 6PGD knockdown (n=3, mean ± SD). **(M)** MHY1485 alleviated the suppressive effect of 6PGD knockdown on cellular viability (n=3, mean ± SD). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.001.

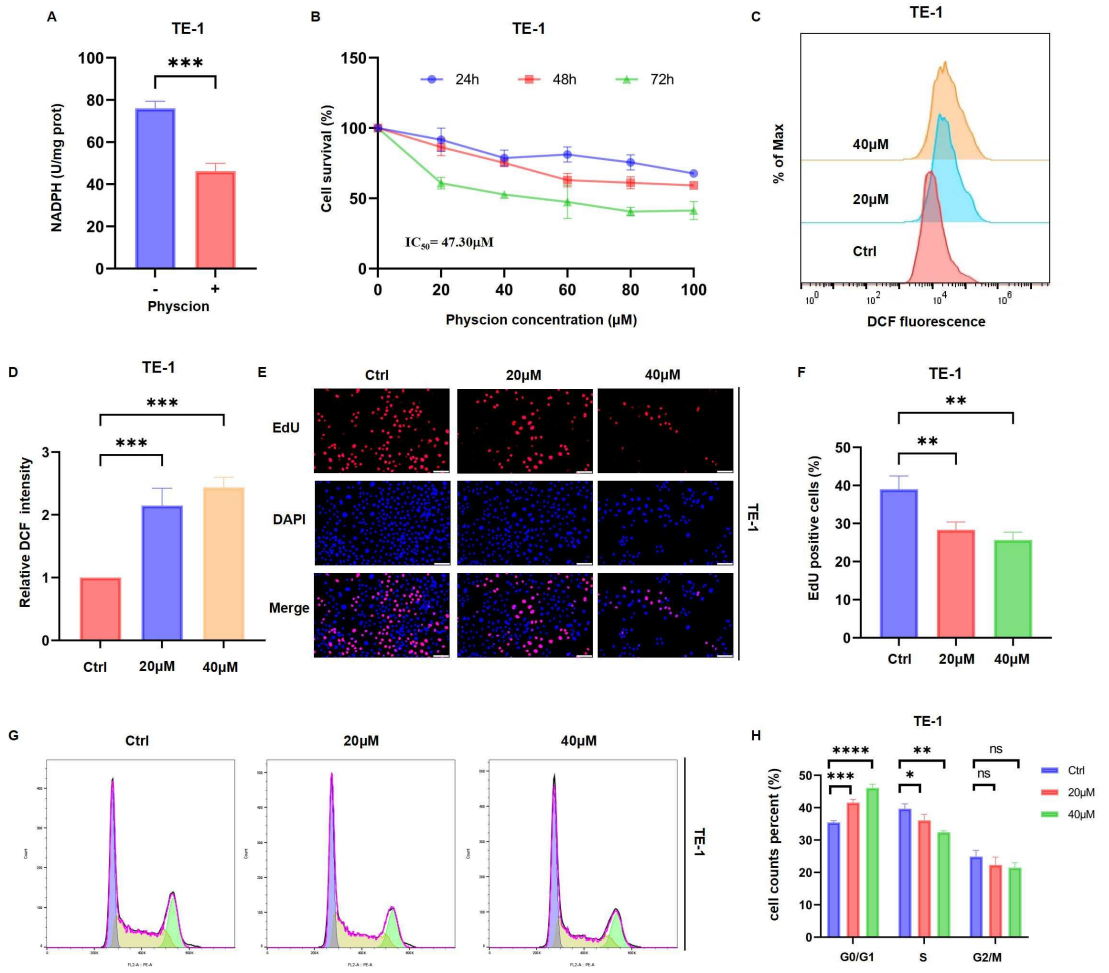


Figure S5. Physcion inhibits the proliferation of TE-1 cells by suppressing 6PGD activity. (A) Physcion (40 μM) significantly reduced the production of NADPH in TE-1 cells (n=3, mean ± SD). **(B)** The cell viability of TE-1 cells was evaluated after treatment with various concentrations of physcion for 24 to 72 hours (n=3, mean ± SD). **(C, D)** The intracellular ROS levels in TE-1 cells fluctuated after 48 hours of physcion treatment (n=3, mean ± SD). **(E, F)** The effect of physcion on DNA synthesis in ESCC cells was detected by EdU staining assay (n=3, mean ± SD); Scale bar: 100 μm. **(G, H)** The cell cycle changes in TE-1 cells was observed after exposure to physcion for 48 hours (n=3, mean ± SD). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; ns means non-significant.

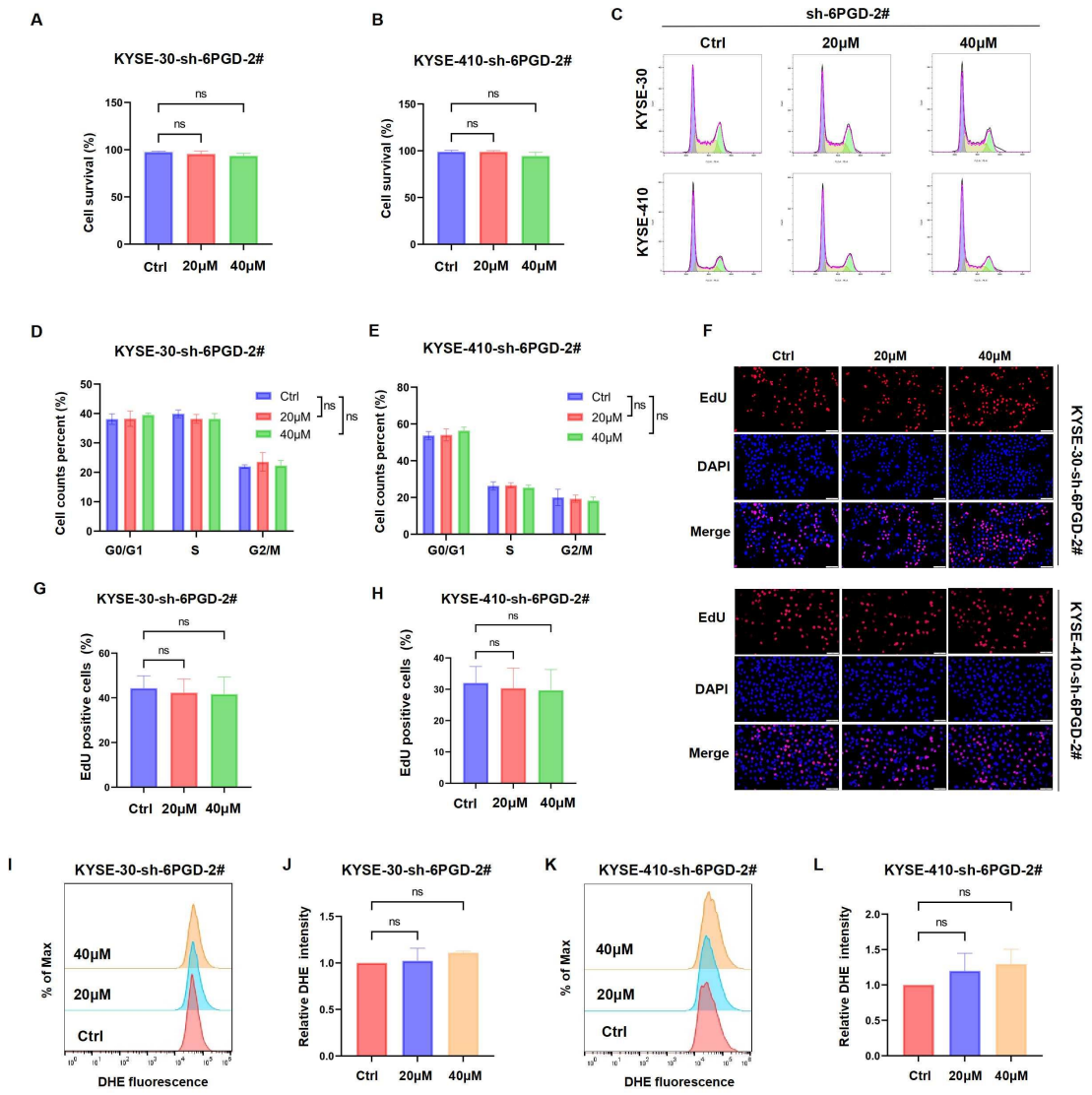


Figure S6. The effect of physcion in 6PGD knockdown cell lines. (A, B) Cell viability was determined using the CCK-8 assay following a 72-hour exposure to physcion (n=3, mean ± SD). **(C-E)** Flow cytometry was employed to analyze the distribution of cells across different phases of the cell cycle after a 48-hour physcion treatment (n=3, mean ± SD). **(F-H)** EdU and DAPI staining were visualized under a fluorescence microscope following a 48-hour physcion treatment (n=3, mean ± SD); Scale bar: 100μm. **(I-L)** Intracellular ROS levels were quantified by flow cytometry after a 48-hour physcion treatment (n=3, mean ± SD). ns means non-significant.

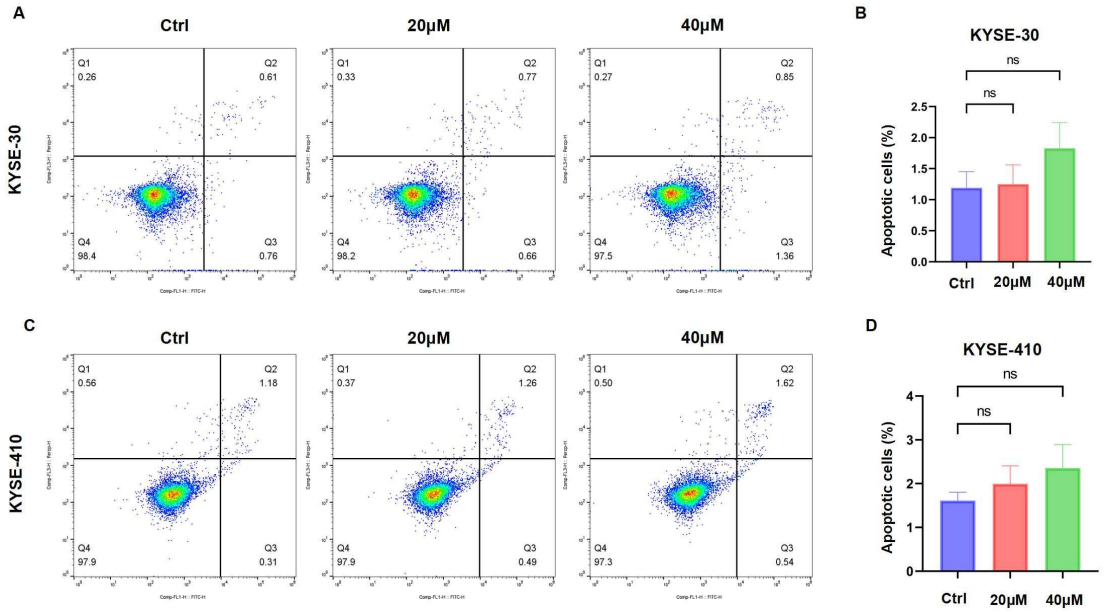


Figure S7. The induction of apoptosis in ESCC cells by physcion appears to be relatively limited. (A, C) Representative images illustrate the apoptosis in KYSE-30 and KYSE-410 cells following exposure to varying concentrations of physcion. **(B, D)** Statistical analysis reveals that the effect of physcion on inducing apoptosis in KYSE-30 and KYSE-410 cells is not statistically significant (n=3, mean ± SD). ns means non-significant.

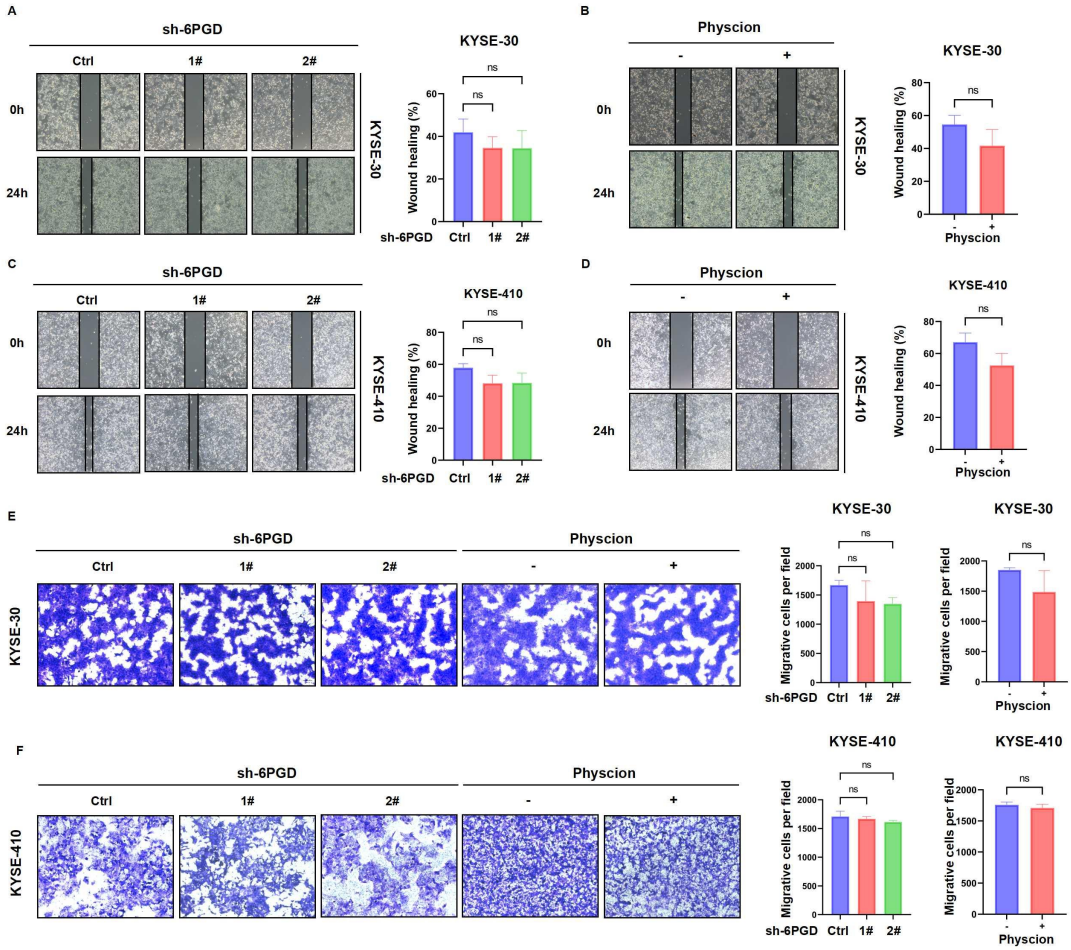


Figure S8. Effect of 6PGD knockdown or physcion treatment on the migration of ESCC cells. **(A, C)** The wound healing assay showed that knocking down 6PGD did not significantly inhibit the migration of KYSE-30 and KYSE-410 cells (n=3, mean ± SD). **(B, D)** Exposure of KYSE-30 and KYSE-410 cells to physcion (40 μM) did not result in a statistically significant alteration in wound healing (n=3, mean ± SD). **(E, F)** The transwell assay demonstrated that neither the knockdown of 6PGD nor the treatment with physcion (40 μM) significantly influenced the migratory capacity of KYSE-30 and KYSE-410 cells (n=3, mean ± SD). ns means non-significant.

Table S1 Univariate and multivariate analyses of survival in ESCC patients (n=180)

Factors	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Gender (Female/Male)	1.005	0.673-1.499	0.982			
Age (≥65/<65)	1.829	1.245-2.686	0.002	1.558	1.048-2.316	0.028
BMI (<23.9/≥23.9)	2.197	1.482-3.258	<0.001	2.312	1.549-3.453	<0.001
Tumor location (Baseline，upper)			0.177			
Middle	1.646	0.951-2.847	0.075			
Lower	1.305	0.738-2.307	0.360			
Tumor length（≥5cm/<5cm）	1.064	0.729-1.555	0.747			
Differentiation (Baseline，well)			0.005			0.027
Moderate	1.252	0.775-2.022	0.358	1.075	0.660-1.752	0.771
Poor	2.115	1.316-3.400	0.002	1.825	1.121-2.970	0.015
T classification (T3-T4/T1-T2)	1.798	1.219-2.653	0.003	1.843	1.238-2.745	0.003
N classification (N0/N1-N3)	2.354	1.590-3.486	<0.001	2.373	1.580-3.564	<0.001
6PGD expression (High/low)	1.838	1.257-2.686	0.002	1.983	1.326-2.965	0.001

Abbreviations: ESCC, esophageal squamous cell carcinoma; HR, hazard ratio; CI, confidence interval; BMI, body mass index; 6PGD, 6-phosphogluconate dehydrogenase.

Table S2 CI values for various concentrations of metformin in combination with physcion in ESCC cells

Physcion (μM)	Metformin (mM)	KYSE-30		KYSE-410	
		Fa	CI value	Fa	CI value
20	1	0.692	0.50446	0.400	0.57828
40	1	0.503	0.35954	0.326	0.77891
60	1	0.396	0.29905	0.231	0.67148
80	1	0.298	0.24865	0.177	0.60893
100	1	0.217	0.20744	0.133	0.51645

Abbreviations: ESCC, esophageal squamous cell carcinoma; Fa, fraction affected, CI, combination index.