Cancer Letters 450 (2019) 22–31

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Metformin induces human esophageal carcinoma cell pyroptosis by targeting the miR-497/PELP1 axis

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ARTICLE INFO

Keywords:
Metformin
Pyroptosis
GSDMD
miR-497
Chemotherapy resistance

ABSTRACT

Evasion of apoptosis is a major contributing factor to the development of chemo- and radiotherapy resistance. Therefore, activation of non-apoptotic programmed cell death (PCD) could be an effective alternative against apoptosis-resistant cancers. In this study, we demonstrated *in vitro* and *in vivo* that metformin can induce pyroptosis, a non-apoptotic PCD, in esophageal squamous cell carcinoma (ESCC), a commonly known chemo-refractory cancer, especially at its advanced stages. Proline-, glutamic acid- and leucine-rich protein-1 (PELP1) is a scaffolding oncogene and upregulated PELP1 in advanced stages of ESCC is highly associated with cancer progression and patient outcomes. Intriguingly, metformin treatment leads to gasdermin D (GSDMD)-mediated pyroptosis, which is abrogated by forced expression of PELP1. Mechanistically, metformin induces pyroptosis of ESCC by targeting miR-497/PELP1 axis. Our findings suggest that metformin and any other pyroptosis-inducing reagents could serve as alternative treatments for chemo- and radiotherapy refractory ESCC or other cancers sharing the same pyroptosis mechanisms.

1. Introduction

Human esophageal squamous cell carcinoma (ESCC) is a devastating malignancy and represents one of the leading causes of gastrointestinal cancer-related death. Similar to most of the other solid cancers that surgery is an effective therapeutic strategy for early-stage ESCC, chemo- and radio-therapies are the main treatment options for ESCC at their advanced stages [1,2]. However, most ESCC will eventually become refractory to these therapies largely due to the development of resistance to apoptosis [3]. Therefore, activation of non-apoptotic programmed cell death (PCD) could be an effective alternative against apoptosis-resistant cancers [4,5] including ESCC.

Pyroptotic cell death or pyroptosis is a non-tradition PCD. Different from apoptosis and more like necrosis, pyroptosis is featured with pore-formation on the plasma membrane which subsequently leads to cell swelling and plasma membrane disruption [6]. It is well established that pyroptosis plays important roles in host defense during pathogen infection [7]. Since gasdermin D (GSDMD) plays an essential role in pyroptotic cell death, pyroptosis is also known as “gasdermin-mediated programmed necrotic death” [5,8–12]. Gasdermins are physiological

Abbreviations: PCD, programmed cell death; ESCC, esophageal squamous cell carcinoma; PELP1, Proline-, glutamic acid- and leucine-rich protein-1; IL-1b, Interleukin 1 Beta; IL-18, Interleukin 18; GSDMD, gasdermin D; ER, Estrogen receptor; ER-α, estrogen receptor-α; ER-β, estrogen receptor-β; HDACs, Histone Deacetylases; KDM1, Lysine Demethylase 1A; PRMT6, Protein Arginine Methyltransferase 6; CARM1, Coactivator-Associated Arginine Methyltransferase 1; FBS, fetal bovine serum; RTCA, real-time cell analyzer system; GEO, Gene Expression Omnibus; GSEA, Gene set enrichment analysis; HRs, hazard ratios; CIs, confidence intervals; IHC, immunohistochemistry; ROC, receiver operating characteristic; RFS, relapse-free survival; OS, overall survival

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https://doi.org/10.1016/j.canlet.2019.02.014
Received 4 December 2018; Received in revised form 1 February 2019; Accepted 8 February 2019

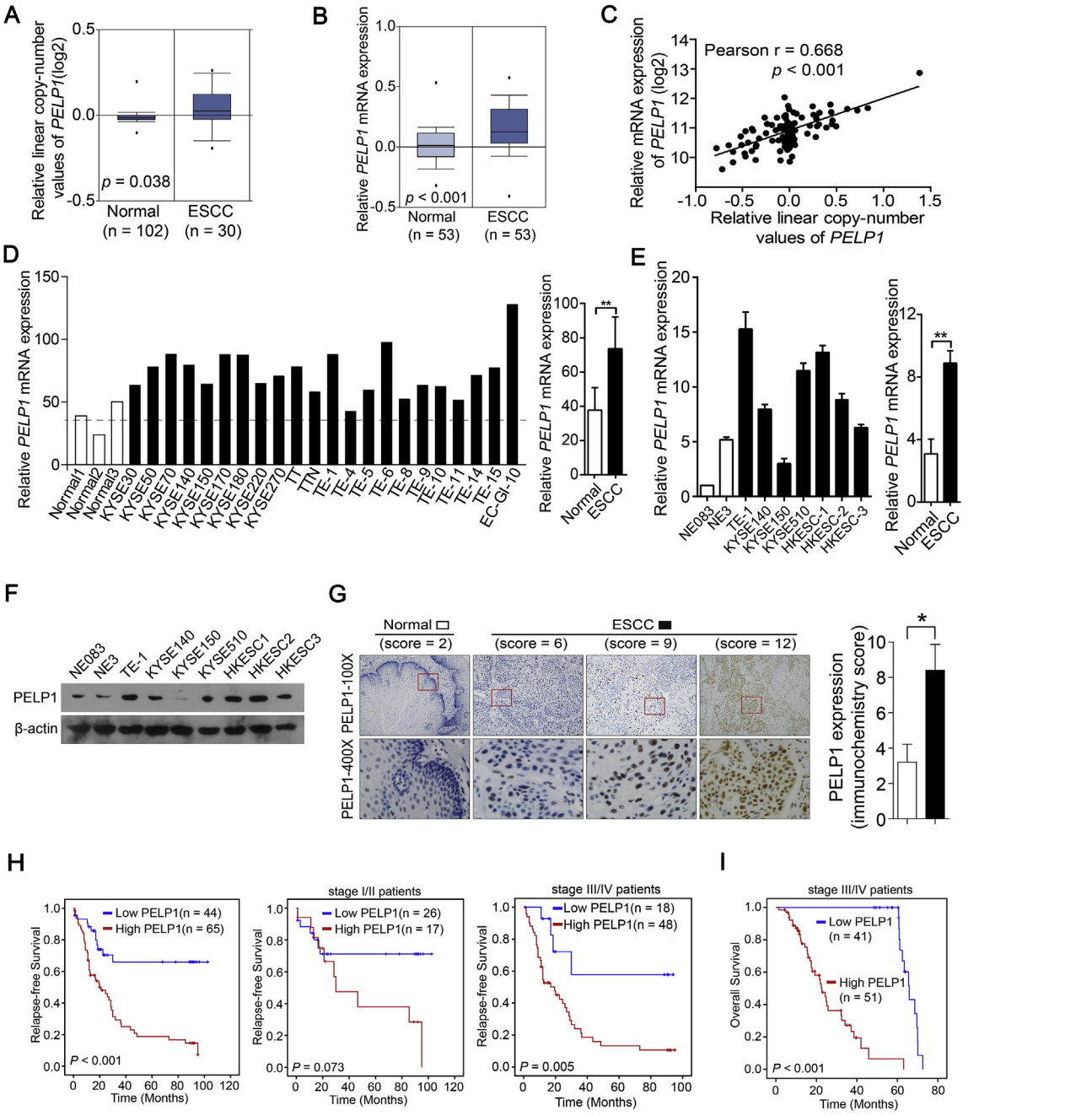

Fig. 1. PELP1 is overexpressed in ESCC patients and associated with poor survival. (**A**) DNA copy number of PELP1 in ESCC was analyzed using Oncomine (www.oncomine.org). (**B**) mRNA levels of PELP1 in ESCC specimens were analyzed using Oncomine. (**C**) The correlation between PELP1 copy number and PELP1 mRNA expression was analyzed by the CbioPortal database (http://cbioportal.org). (**D**, Left) mRNA levels of *PELP1* in a panel of ESCC cell lines (filled bars) and immortal normal esophageal epithelial cell lines (open bar) were analyzed in the ESCC dataset from GEO, GSE63941. (Right) The expression value of PELP1 in ESCC cell lines (filled bar) and immortal normal esophageal epithelial cell lines (open bar) was plotted. (**E**, Left) RT-qPCR analysis of PELP1 mRNA level in a panel of ESCC cell lines (filled bars) and immortal normal esophageal epithelial cell lines (open bar). (Right) The expression value of PELP1 in 7 ESCC cell lines (filled bar) and 2 immortal normal esophageal epithelial cell lines (open bar) was plotted. (**F**) Western blot analysis of PELP1 in 7 ESCC cell lines and 2 immortal normal esophageal epithelial cell lines. β-actin was used as an internal control. (**G**, Left) Immunohistochemical staining of PELP1 (brown) on ESCC sections (n = 109) and paired adjacent noncancerous tissues. Nuclei were counterstained with hematoxylin (blue). (Magnification: Top, 100 × ; Bottom, 400 ×). (Right) The immunohistochemistry score of PELP1 in ESCC (filled bar) and paired adjacent noncancerous tissues (open bar) tissues was plotted. (**H**) Kaplan–Meier curves for relapse-free survival (RFS) among all-stage patients (left panel), stage I/II patients (middle panel), or stage III/IV patients (right panel) with high and low protein levels of PELP1. (I) The relationship between overall survival and mRNA levels of *PELP1* in a cohort of advanced-stage (stage III/IV) patients from a GEO ESCC Dataset (GSE53625) was analyzed by Kaplan–Meier survival analysis. Error bars indicate SD. $*p < 0.05$, $**p < 0.01$ by Student's t-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

substrates of inflammatory Caspase 1. Cleavage of GSDMD and subsequent pyroptotic cell death is an effective option to rid of bacterial infection [13]. More recent findings suggest that GSDMD can serve as a substrate for multiple inflammatory caspases including caspase-1, 4, 5, 11 [9]. Mechanistically, following dangerous signals such as microbial infection and/or endogenous damage-associated signals [8,12], inflammasomes are activated and GSDMD is cleaved yielding an N-terminal fragment of GSDMD (GSDMD-N). Subsequently, GSDMD-N

interacts with membrane phospholipids to form pores in the plasma membrane, which eventually leads to pyroptosis. GSDMD-mediated pyroptosis is also accompanied by inflammatory cytokines IL-1b and IL-18 releasing [14]. Therefore, GSDMD cleavage is a signature character of pyroptosis [9]. Although it is well known that inflammatory responses play important roles in the development of cancers including ESCC and pyroptosis is closely associated with inflammation, little attention has been paid to the roles of pyroptosis and their application in cancer therapy.

Metformin is a widely used anti-diabetic medication. A large body of evidence indicates that metformin also possesses anti-cancer properties. This makes metformin not only a preventive reagent but also a potential therapeutic agent in cancer treatment [15]. We have reported that metformin can inhibit both growth and progression of ESCC [16]. Multiple lines of evidence suggest that metformin's anti-cancer effects are based on distinct mechanisms including the induction of cell cycle arrest, apoptosis, autophagy, and senescence [16–18]. More recent evidence suggests that the anti-cancer effects of metformin are through regulating the expression of different miRNAs [19]. However, the exact underlying mechanisms of metformin's anti-cancer effects remain incompletely defined.

PELP1 is a transcriptional coregulator [20]. As a scaffolding oncogenic protein and by interacting with different epigenetic modifiers such as HDACs, KDM1, PRMT6, and CARM1, PELP1 is directly involved in the modification of histones [21,22], which generally provide the cancer cells with distinct growth and survival advantages [23,24]. In breast cancer, PELP1 is not only oncogenic but also involves in tamoxifen-resistance and chemoresistance [25,26]. Estrogen receptor (ER) is an unfavorable independent prognostic indicator in ESCC [27]. PELP1 was originally defined as an ER coactivator, later on, was found to be an adaptor protein in histone/chromatin remodeling, involved in many physiological and pathological events. PELP1 has been characterized as an oncogenic coactivator in hormone-response cancer, such as breast cancer [25,28]. Interestingly, metformin can protect against the testosterone-induced elevation of estrogen receptor-α (ER-α) and decreases of estrogen receptor-β (ER-β) expression [29]. However, the roles of PELP1 in metformin-induced effects against cancers remain unknown.

In this study, we first showed that the levels of PELP1 are highly correlated with tumor progression as well as the patient's outcomes. The results from our *in vitro* and *in vivo* studies demonstrated that metformin is capable of inducing ESCC cell pyroptosis. Mechanistically, by upregulating miR-497 metformin downregulates PELP1 and ultimately induces pyroptosis. Thus, we identified an unrecognized mechanism for metformin's anti-cancer effects and these findings imply that metformin could serve as a potential treatment option for cancers resistant to chemo- and radio-therapies but sensitive to pyroptosis.

2. Materials and methods

2.1. Patient specimens

We collected 109 tumor samples of human primary ESCC and their paired adjacent normal tissues in the Affiliated Tumor Hospital of Shantou University Medical College from 2009 to 2017 and embedded in paraffin. All ESCC patients were diagnosed and confirmed histologically. None of them underwent adjuvant chemotherapy or radiation treatment prior to surgery. The study protocol was reviewed and approved by the Ethics Committee of Shantou University Medical College.

3. Results

3.1. PELP1 plays important role in ESCC progression and patient outcome

To systemically study the role of PELP1 in ESCC, we first analyzed its DNA copy number in the Oncomine database (https://www.oncomine.org//) and found that the copy number of *PELP1* in human ESCC is significantly higher than that of the normal esophageal tissues ($p = 0.038$; Fig. 1A) [30]. In addition, analysis of a different set of samples from the Oncomine database which includes 53 ESCC specimens [31] also found that the mRNA levels of *PELP1* were significantly elevated in ESCC compared to their paired adjacent tissues ($p < 0.001$; Fig. 1B). Furthermore, a strong positive correlation between the copy number and the mRNA level of PELP1 in ESCC (Pearson r = 0.668, $p < 0.001$; Fig. 1C) has been identified when the data from the cBioPortal database (www.cbioportal.org) were analyzed, suggesting that gene amplification is at least in part the explanation of PELP1 overexpression in ESCC. In accordance, the mRNA levels of PELP1 in all 22 ESCC cell lines tested are higher than that of the fibroblasts derived from normal esophageal tissues in a published GEO dataset (GSE63941) (Fig. 1D). Then, we examined the mRNA and protein levels of PELP1 in different ESCC cell lines by RT-qPCR and western blotting assays, respectively. Fig. 1E and F shows that compared to that of the immortalized esophageal epithelial cells, both mRNA and protein levels of PELP1 are elevated in all ESCC cells examined. We also compared the protein levels of PELP1 in 109 ESCC patient specimens with their paired adjacent tissues by immunohistochemistry (IHC) and found that the protein levels of PELP1 in ESCC tissues are significantly elevated ($p < 0.05$; Fig. 1G). These data altogether demonstrated that not only the DNA copy number but also the mRNA and protein levels of PELP1 increase in human ESCC.

To determine the clinical importance of PELP1 in human ESCC, we developed a receiver operating characteristic (ROC) curve. Using 5 as the cutoff score for PELP1 overexpression (Supplementary Fig. S1A), we found that PELP1 is overexpressed in 59.6% (65 of 109) of human ESCC specimens. Results from correlation analysis showed that PELP1 overexpression is positively correlated with tumor depth ($p = 0.048$), lymph node metastasis ($p = 0.011$) and staging ($p = 0.001$) (Table 1), implying that PELP1 may play important role in ESCC progression. Kaplan-Meier analysis showed that ESCC patients with PELP1 overexpression have poorer relapse-free survival (RFS) ($p < 0.001$; Fig. 1H, left). More importantly, the correlation between PELP1 expression and poor RFS is significant only in advanced (Stages III/IV) ($p = 0.005$;

Table 1
Demographics and clinicopathological characteristics of ESCC patients.

Variables	Patients: n	PELP1 level		*P*-value
		Low: n (%)	High: n (%)	
Total samples	109	44 (40.4)	65 (59.6)	
Age (years)				
≤ 60	57	27 (47.4)	30 (52.6)	0.171
> 60	52	17 (32.7)	35 (67.3)	
Gender				
Female	29	14 (48.3)	15 (51.7)	0.378
Male	80	30 (37.5)	50 (62.5)	
Tumor depth				
T1/T2	30	17 (56.7)	13 (43.3)	0.048
T3/T4	79	27 (34.2)	52 (65.8)	
Lymph node metastasis				
N0	50	27 (54.0)	23 (46.0)	0.011
N1-N3	59	17 (28.8)	42 (71.2)	
Differentiation				
Well	30	16 (53.3)	14 (46.7)	0.087
Moderate	45	19 (42.2)	26 (57.8)	
Poor	34	9 (26.5)	25 (73.5)	
Tumor size (cm)				
≤ 5	37	17 (45.9)	20 (54.1)	0.416
> 5	72	27 (37.5)	45 (62.5)	
Stage				
I/II	43	26 (60.5)	17 (39.5)	0.001
III/IV	66	18 (27.3)	48 (72.7)	

High in this analysis is based on a PELP1 level > 5; the remaining individuals were classified as low.

Fig. 1H, right), but not the early-stage ESCC (Stages I/II) ($p = 0.073$; Fig. 1H, middle). In accordance, Kaplan-Meier analysis of GEO database containing 179 ESCC patients (GES53625) revealed that ESCC patients at advanced stages (n = 92) harboring overexpressed *PELP1* have worse overall survival (OS) ($p < 0.001$; Fig. 1I), while patients at all and early stages (n = 87) do not ($p = 0.678$ and $p = 0.888$, respectively; Supplementary Figs. S1B and C). These data strongly suggest that PELP1 plays important role in ESCC recurrence and elevated levels of PELP1 in ESCC lead to poor prognosis.

3.2. Metformin inhibits ESCC cell proliferation via downregulating PELP1

Therapeutic resistance in advanced cancers is not only common but also the main cause of recurrence [32,33]. Since the importance of elevated PELP1 on cancer recurrence and prognosis was particularly observed in patients with advanced ESCC, we speculated that PELP1 may be involved in ESCC chemotherapy resistance which is largely attributed to evading apoptosis [4,5]. PELP1 is an ER coactivator and metformin can affect ER expression. We decided to determine the effect of metformin on PELP1 experimentally. Two ESCC cell lines, KYSE510 and KYSE140, were treated with different concentrations of metformin (0, 1, 5, 10 and 20 mM), and both the protein and mRNA levels of PELP1 were downregulated by metformin in a dose-dependent manner (Fig. 2A). When the cells were treated with 10 mM of metformin for different periods (24, 48 and 72 h), the levels of both protein and mRNA levels of PELP1 were downregulated in a time-dependent manner (Fig. 2B). To determine whether PELP1 plays a key role in metformin-mediated cell growth, ESCC cells with or without forced expression of PELP1 were treated with metformin (Fig. 2C) and real-time proliferation assays were conducted. We found that the inhibitory effect of metformin on ESCC cells proliferation start to appear at 40 h following metformin treatment and peaked at 60 h (Fig. 2D, upper panel). Of note, overexpression of PELP1 alone was able to increase ESCC cells proliferation dramatically (Fig. 2D, middle panel), supporting the oncogenic role of PELP1 in ESCC. More importantly, overexpression of PELP1 largely attenuated the inhibitory effects of metformin-inhibited cell growth (Fig. 2D, lower panel). These data suggest that metformin represses ESCC cell proliferation by downregulating PELP1.

3.3. Metformin induces pyroptosis via downregulating PELP1

When we took a closer look of the ESCC cell treated with metformin under a light microscope, we noticed that some cells exhibited distinct morphology characterized with large bubbles derived from the plasma membrane and the cells appear to be swelled (Fig. 3A). Since these morphologic changes resemble pyroptosis [9,14], we decided to exam whether metformin can induce ESCC cell pyroptosis. First, we treated the two ESCC cell lines (KYSE510, KYSE140) with 10 mM metformin for 48 h and examined the localization of GSDMD. The results showed that metformin treatment leads GSDMD translocation from the cytoplasm to the cell membrane as evidenced by immunofluorescence (Fig. 3B and Supplementary Fig. S2B). Immunoblotting results also demonstrated that metformin treatment induced GSDMD cleavage (Fig. 3C and Supplementary Fig. S2C). Since gene amplification of *GSDMD* was found in 17.71% of human ESCC (n = 96) (cBioPortal database, Fig. 3D) and the mRNA levels of *GSDMD* in human ESCC tissues were relatively elevated (Oncomine database, $p < 0.001$, Fig. 3E, left panel; UALCAN database, http://ualcan.path.uab.edu, $p < 0.001$, Fig. 3E, right panel), we then conducted immunohistochemistry (IHC) of TMA consisting of primary ESCC specimens along with their paired adjacent normal tissues and found that the levels of GSDMD in human ESCC is elevated ($p < 0.001$, Fig. 3F). Interestingly, the mRNA levels of *Caspase 1* were also elevated in human ESCC tissues (Oncomine database, $p = 0.002$, Supplementary Fig. S2A, upper panel; UALCAN, $p < 0.001$, Supplementary Fig. S2A, bottom panel). To determine if PELP1 plays any role in metformin-induced pyroptosis, we conducted a GSEA analysis and found that PELP1 is inversely associated with GSDMD (GSE23400, Fig. 3G, middle panel), Caspase 1 (Fig. 3G, left panel), and NLRP3 inflammasome signature (GSE20347, Fig. 3G, right panel). More importantly, overexpressed PELP1 in ESCC cells is not only capable of inhibiting metformin-induced GSDMD cytoplasm-to-membrane translocation (Fig. 3H) but also GSDMD cleavage (Fig. 3I). These data suggest that PELP1 inhibits ESCC cell pyroptosis and metformin enhances ESCC pyroptosis by downregulating PELP1.

3.4. Metformin downregulates PELP1 through upregulating miR-497

Multiple lines of evidence imply that metformin can function through different miRNAs [19,34]. To determine if metformin-downregulated PELP1 is mediated by miRNAs, we conducted a bioinformatics analysis (http://www.targetscan.org/vert_72/) to identify potential miRNAs involved in PELP1 regulation and found six miRNAs (Fig. 4A) potentially interact with the 3′UTR of *PELP1* mRNA. Further analysis of these miRNAs in human ESCC tissues versus the normal tissues (GSE43732 and TCGA) found that two of them (miR-195 and miR-497) were highly downregulated and the other four were upregulated in cancer tissues (Fig. 4B and C). To determine if any of these miRNAs were involved in metformin-mediated PELP1 downregulation, we treated the KYSE510 cells with different concentrations of metformin (0, 5 and 10 mM) and estimated the levels of these miRNAs. Fig. 4D showed that metformin upregulated miR-497 dramatically and miR-195 moderately, while the other four miRNAs were not affected by metformin treatment ($p < 0.01$). We then focused our attention on miR-497 for the rest of the research.

To determine the role of miR-497 in PELP1 expression, miR-497 mimic was transiently transfected into KYSE510 cells and the mRNA levels and protein levels of PELP1 were estimated by RT-qPCR and western blot, respectively. Fig. 4E showed that comparing to the negative control miR-497 mimic is capable of downregulating PELP1 significantly ($p < 0.01$). In addition, we constructed luciferase reporters harboring the 3′-UTR of PELP1 with either wildtype (Wt) or mutant (Mut) miR-497 binding site. The KYSE510 cells were transiently co-transfected with miR-497 mimic and reporter plasmid and luciferase activities were measured. Fig. 4F showed that miR-497 only inhibited the luciferase activity of the Wt reporter ($p < 0.01$), suggesting that in ESCC cells the PELP1 is targeted by miR-497. To determine the role of miR-497 in metformin-downregulated PELP1 in ESCC cells, KYSE510 cells transfected with or without miR-497 inhibitor were treated with or without metformin (10 mM) for 48 h, and the mRNA levels and protein levels of PELP1 were estimated by RT-qPCR and western blot. Fig. 4G showed that inhibiting miR-497 leads to higher level of PELP1, and metformin can counteract miR-497 inhibitor-induced PELP1 upregulation. These data demonstrated an indispensable role of this miRNA in metformin-downregulated PELP1. These data altogether demonstrated that in ESCC cells metformin upregulates miR-497 which in turn downregulates PELP1 and ultimately leads to pyroptosis.

3.5. Metformin induces ESCC pyroptosis by targeting miR-497/PELP1 axis in an animal model

To demonstrate that metformin is capable of inducing ESCC cell pyroptosis by targeting the miR-497/PELP1 axis *in vivo*, we established an ESCC animal model by inoculating human ESCC cells (KYSE510) into immune-deficient mice. A week after the inoculation, we started to treat the mice for 4 weeks with a daily intraperitoneal injection of metformin (250 mg/kg body weight) and tumor tissues were harvested and processed upon scarification of the mice at the end of the treatment (Fig. 5A). Fig. 5B showed that both the size and weight of the tumors were significantly reduced in mice treated with metformin. In addition, RT-qPCR results showed that the mRNA levels of PELP1 and miR-497 were down- and up-regulated by metformin, respectively (Fig. 5C and

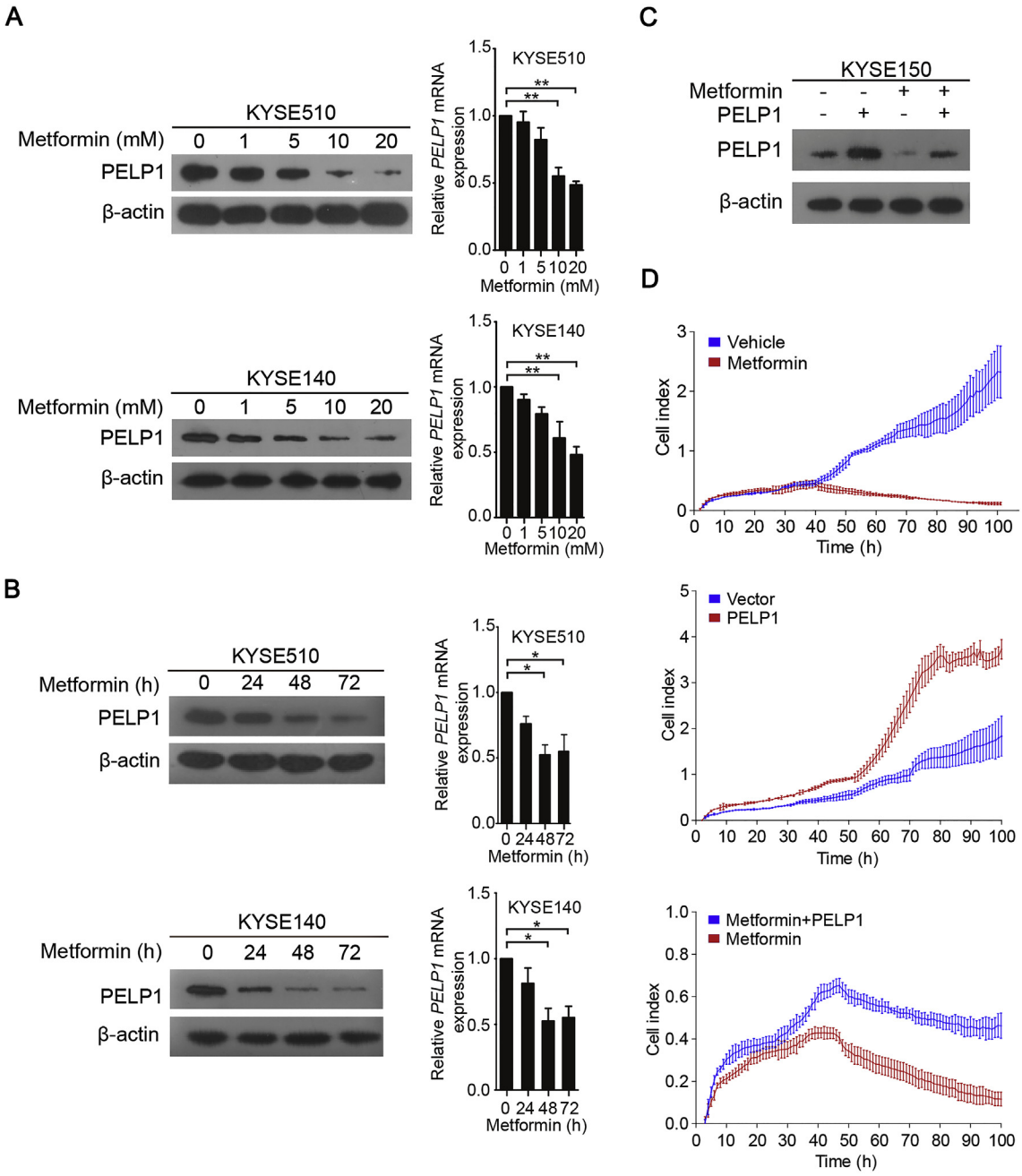

Fig. 2. Metformin targets PELP1 in ESCC cells. (**A**) KYSE510 and KYSE140 cells that treated with metformin (1, 5, 10, or 20 mM) or vehicle solution for 48 h were subjected to Western blot (left) and RT-qPCR (right) analysis of PELP1 expression. (**B**) KYSE510 and KYSE140 cells were treated with 10 mM metformin or vehicle solution for the indicated times (0, 24, 48, or 72 h) and then analyzed by Western blot (left) and RT-qPCR (right) analysis for PELP1 expression. (**C**) KYSE150 cells transfected with or without PELP1 overexpression plasmid were treated with or without metformin (10 mM) for 48 h and then subjected to Western blot for PELP1 expression. β-actin was used as an internal control. (**D**) KYSE150 cells were treated with 10 mM metformin alone, or transfected with PELP1 overexpression plasmid alone, or co-treated with metformin and PELP1 overexpression plasmid transfection, for 48 h, and then the proliferation of these cells was monitored by RTCA for 100 h. Error bars indicate SD. $*p < 0.05$, $**p < 0.01$ by a one-way ANOVA with post hoc intergroup comparisons (B and C).

D). Consistently, both IHC and immunoblotting showed that the levels of PELP1 in tumors carried by metformin-treated mice reduced dramatically (Fig. 5E and F). More importantly, immunoblotting demonstrated that the levels of cleaved GSDMD in the tumor tissues also increased significantly in mice treated with metformin (Fig. 5F). These findings altogether demonstrated that metformin is capable of inducing ESCC cell pyroptosis *in vivo* by targeting the miR-497-PELP1 axis.

4. Discussion

Unlike apoptosis, pyroptosis represents a distinct type of programmed cell death which usually initiated by infections of bacteria, fungi, and parasite [9]. However, similar to apoptosis that pyroptosis ultimately leads to cell death. Therefore, induction of pyroptosis can be considered as an alternative mechanism in treating cancers resistant to apoptosis [35]. Our *in vitro* and *in vivo* data demonstrated that metformin is capable of inducing ESCC cell pyroptotic death evidenced by the elevated levels of cleaved GSDMD in metformin-treated cells.

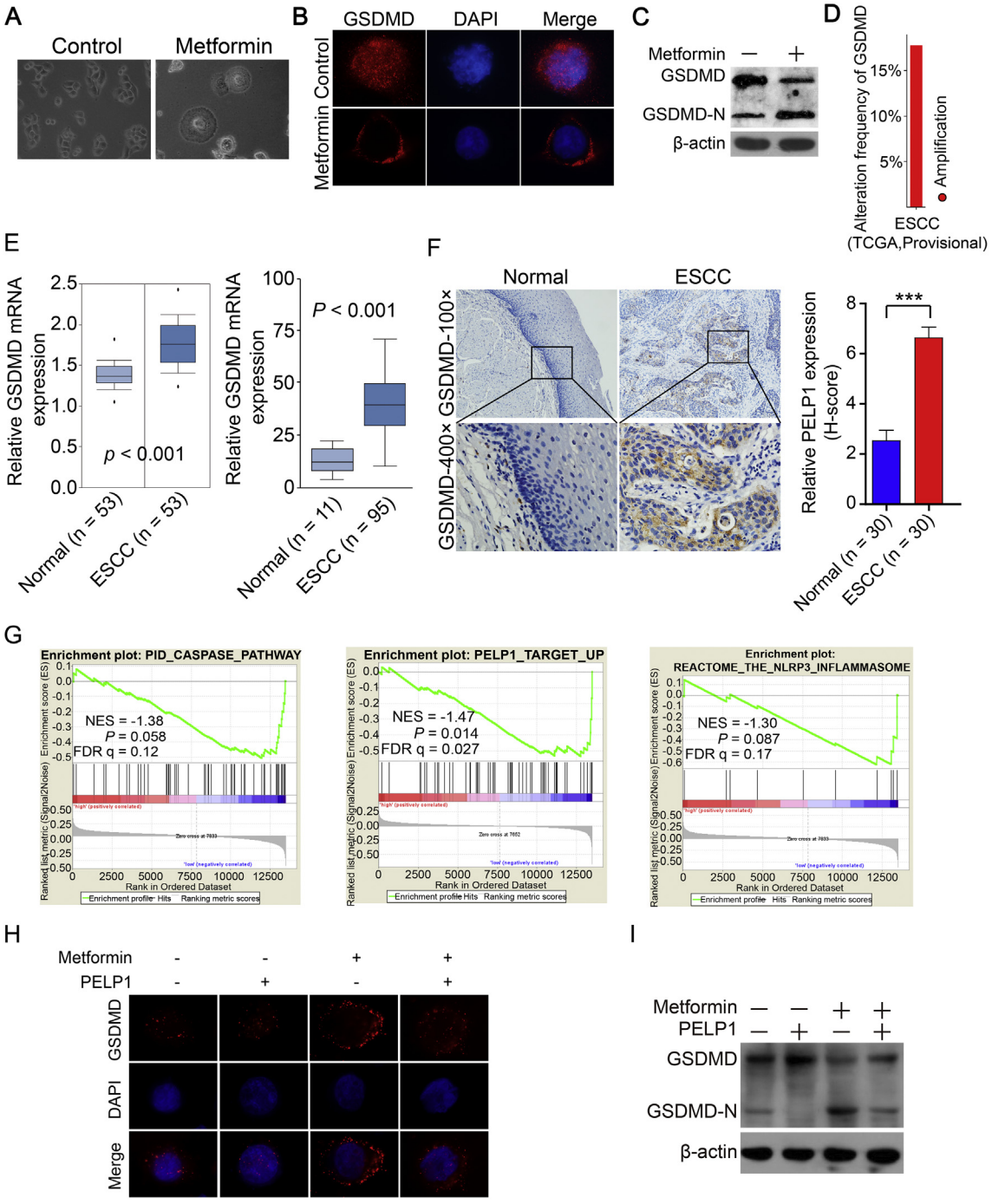

Fig. 3. Metformin induces pyroptosis cell death via PELP1 in ESCC cells. (**A**) Phase-contrast images of KYSE510 cells morphology that treated with 10 mM metformin or vehicle solution for 48 h. (**B**) Immunofluorescence assay of GSDMD (Red) in KYSE510 cells that treated with 10 mM metformin or vehicle solution for 48 h. Nuclei were stained with DAPI (blue). (**C**) Western blot analysis of metformin-induced GSDMD cleavage. (**D**) Alteration of GSDMD in ESCC (n = 96) was analyzed using CbioPortal database. (**E**) The expression of GSDMD was investigated in Oncomine database (left panel) and UALCAN database (http://ualcan.path.uab.edu) (right panel). (**F**) Immunohistochemical staining of GSDMD (brown) on ESCC sections (n = 30) and paired noncancerous tissues. The immunohistochemistry score of GSDMD in ESCC (red bar) and paired adjacent noncancerous tissues (blue bar) tissues was plotted. (**G**) Gene set enrichment analysis (GSEA) showing inverse correlations between PELP1 expression and the Caspase pathway (PID_CASPASE_PATHWAY) (left panel), and inverse correlation between GSDMD expression and PELP1 signature (PELP1_TARGET_UP) in a published cohort of ESCC patients (GSE23400, n = 53) (middle panel), and inverse correlation between PELP1 expression and the NLRP3 inflammasome signature (REACTOME_THE_NLRP3_INFLAMMASOME) in a published cohort of ESCC patients (GSE20347, n = 17) (right panel). (**H**) Immunofluorescence assay of GSDMD (Red) in KYSE150 cells that transfected with PELP1 overexpression plasmid or control vector treated with 10 mM metformin or vehicle solution for 48 h. Nuclei were stained with DAPI (blue). (**I**) Western blot analysis of GSDMD cleavage in KYSE150 cells that transfected with PELP1 overexpression plasmid or control vector treated with 10 mM metformin or vehicle solution for 48 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

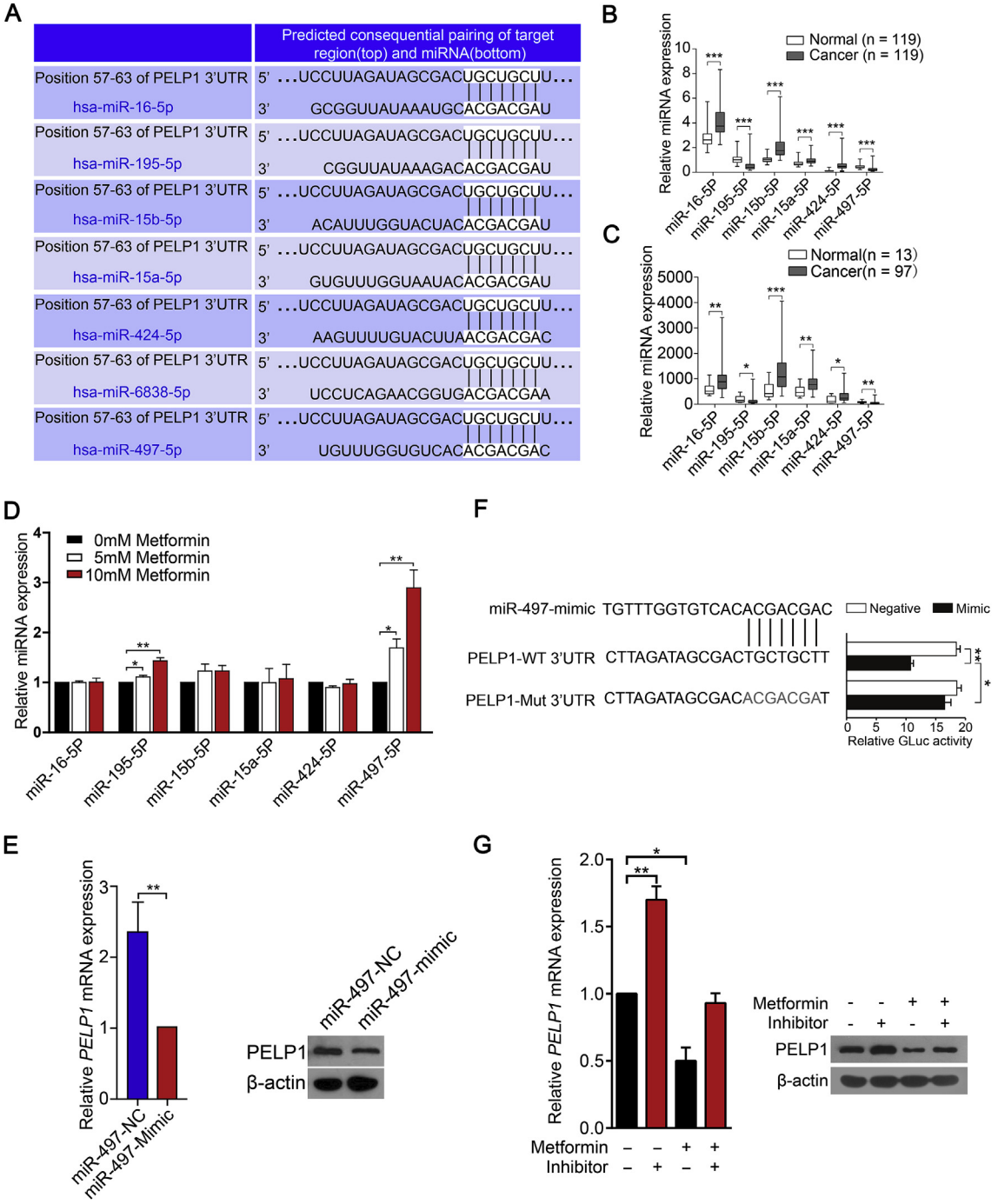

Fig. 4. Metformin inhibits PELP1 through induction of miR-497. (**A**) The Target Scan database (http://www.targetscan.org/vert_72/) predicts 7 putative miRNA binding sites on the 3′-UTR of PELP1. (**B** and **C**) The expressions of the 6 out of 7 putative miRNAs were analyzed in an ESCC dataset from GEO (GSE43732), which includes 119 ESCC and paired adjacent normal tissues (B), and in a TCGA dataset (titled "ESCA"), which includes 97 ESCC and 13 normal esophageal tissues (C). (**D**) RT-qPCR analysis of 6 putative PELP-targeting miRNAs in KYSE510 cells treated with 10 mM metformin or vehicle solution for 48 h. (**E**) RT-qPCR and immunoblot analysis of miR-497 in KYSE510 cells treated with miR-497 mimic or negative control. (**F**) The luciferase reporter plasmid containing wild-type or mutant PELP1 3′-UTR was co-transfected into KYSE510 cells with miR-497 mimic. Luciferase activity was determined after 48 h transfection. (**G**) KYSE510 cells transfected with or without miR-497 inhibitor were treated with or without metformin (10 mM) for 48 h and then subjected to RT-qPCR and Western blot for PELP1 expression. β-actin was used as an internal control. Error bars indicate SD. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by Student's t-test.

Mechanistically, by upregulating miR-497, metformin downregulates the levels of PELP1 and ultimately leads to ESCC cell pyroptosis. Given that (1) PELP1 is highly expressed in both ESCC cell lines and patients' ESCC tissues (Fig. 1G); (2) the levels of PELP1 are positively associated with clinical outcomes including tumor depth, lymph node metastasis, staging and more importantly PELP1 overexpression is accompanied by poorer relapse-free survival; and (3) metformin is capable of inducing ESCC cell pyroptosis by targeting the miR-497/PELP1 axis in animal model, we propose that metformin and any reagents capable of inducing pyroptosis can be developed as therapeutic strategies for ESCC treatments.

Although the levels of PELP1 affect relapse-free survival in general, we found that the correlation between PELP1 expression and poor RFS is significant only in advanced but not the early-stage ESCC (Fig. 1H).

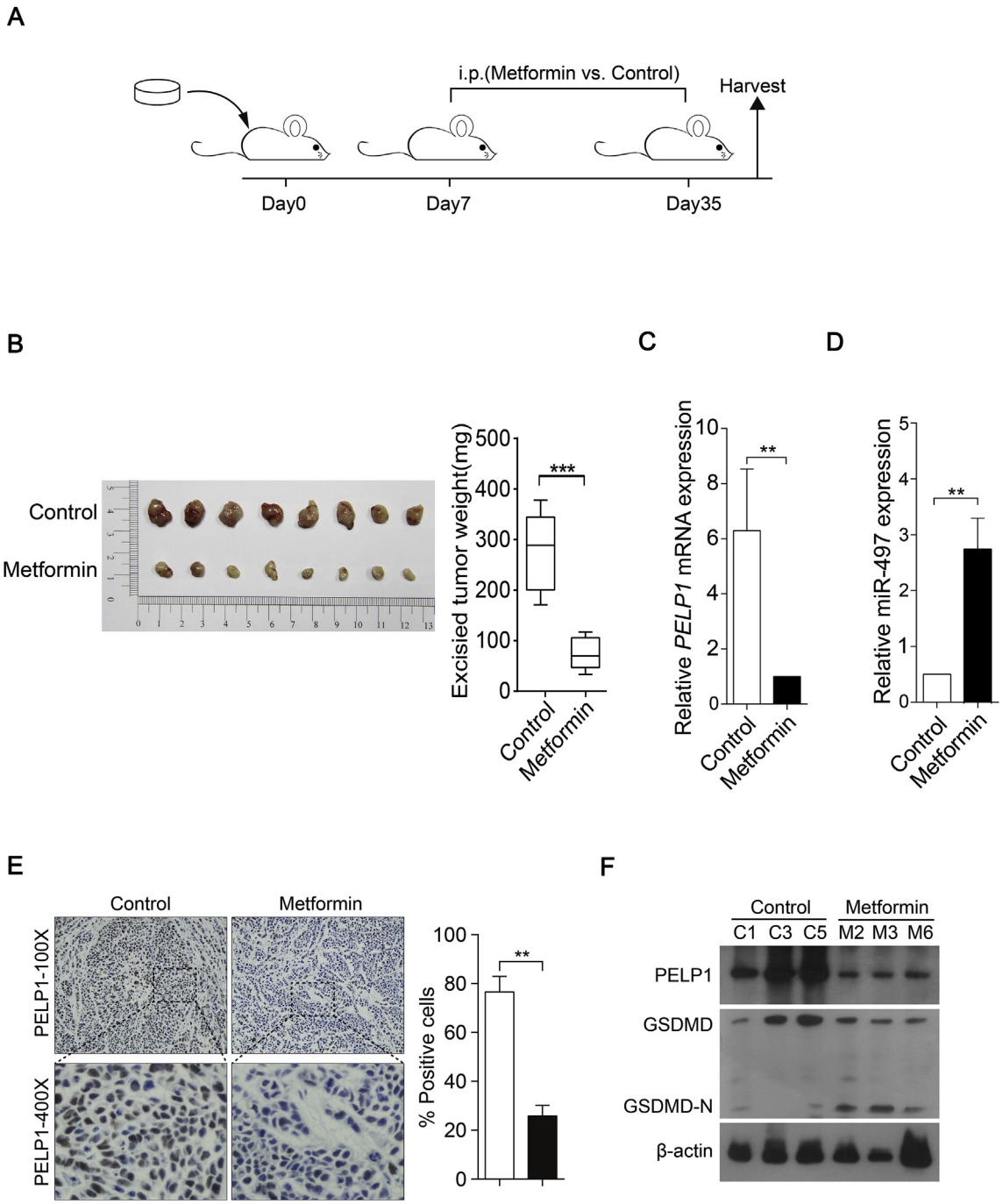

Fig. 5. Metformin induces pyroptosis *in vivo* and regulates the expression of miR-497 and PELP1 *in vivo*. (**A**) Schematic model of metformin treated-mice: KYSE510 cells were injected into flanks of nude mice, after 1 week, mice models (n = 8 in each group) were treated with metformin administration or vehicle. (**B**) Xenografted tumors were harvested at the end of the experiments (left). The average weights of tumors derived from treatment with metformin or vehicle (right). (**C** and **D**) RT-qPCR analysis of PELP1 mRNA (C) and miR-497 (D) in tumors derived from mice that treated with metformin or vehicle. (**E**) Immunohistochemical staining of PELP1 (brown) on tumor sections derived from treatment with metformin or vehicle. Nuclei were counterstained with hematoxylin (blue). (Magnification: Top, 100 × ; Bottom, 400 ×) (left). The percentage of PELP1-positive cells in tumor sections from treatment with metformin is plotted against that observed in the vehicle (right). (**F**) Western blot analysis of GSDMD cleavage in tumors derived from treatment with metformin or vehicle. Error bars indicate SD. $^{**}p < 0.01$, $^{***}p < 0.001$ by Student's t-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

This finding is in accordance with the results from Kaplan-Meier analysis of GEO database, which showed that ESCC patients at their advanced stages not only have higher levels of PELP1 but also with shorter OS (Fig. 1I). Given that cancer recurrence is largely attributed to chemo-radiation resistance [1,2] and PELP1 has been implied in tamoxifen resistance [26] and chemoresistance in breast cancer [36], we speculate that PELP1 could play important role in the development of chemo- and radiation resistance in ESCC. PELP1 has been reported to be involved in the regulation of programmed cell death including apoptosis and autophagy [28,37]. Here, we showed that PELP1 is capable of inducing pyroptosis. Given that PELP1 is a transcriptional coregulator, we have also attempted to determine if PELP1 regulates GSDMD transcriptionally. However, manipulation of PELP1 expression has no effect on the mRNA level of GSDMD (Supplementary Figs. S3A–D) suggesting

that PELP1 mediates pyroptosis through mechanisms other than regulating GSDMD transcription. Given the fact that PEPL1, as a scaffolding protein, can interact with both histone and methyltransferase CARM1 [22,38], we also speculate that PELP1 could be involved in the regulation of pyroptosis epigenetically.

Although the physiological relevance of pyroptosis in cancers has been assumed, the mechanisms were far from clear and sometimes the findings from different studies were controversial. For example, Omega-3 and Simvastatin were reported to exert their anti-tumor effects through inducing pyroptosis in triple negative breast cancer and non-small cell lung cancer, respectively [39,40]. GSDME, another member of gasdermin family, is expressed in various normal tissues but is silenced in most tumor cells [5]. Since chemotherapy induces pyroptosis and apoptosis in GSDME-positive and GSDME-negative cells, respectively, the authors suggested that the level of gasdermin is the determinant of the type of programmed cell deaths [5]. Although both GSDME and GSDMD are involved in pyroptosis, GSDME is activated by caspase3 and GSDMD is cleaved by caspase1/4/5/11 [9,41]. In addition, GSDMD was reported to be downregulated in gastric cancer [42]. Our systemic study found that the *GSDMD* gene is amplified and both mRNA and protein levels of GSDMD were elevated in ESCC. In summary, we found that PELP1 plays important roles in ESCC cancer recurrence and prognosis. Metformin can induce GSDMD-mediated ESCC cell pyroptosis by targeting the miR-497-PELP1 axis. Our findings suggest that metformin and any other pyroptosis-inducing reagents possess great potential in treating chemo- and radiation resistant ESCC.

Author contributions

H.Z. conceived and designed the experiments, interpreted data and wrote the manuscript. L.W., K.L., X.L., Z.Y., and S.W. performed the *in vitro* experiments patient specimen analysis, bioinformatics assay and analyzed data. X.X., Z.N., J.W. and X.X. contributed to research data; Y.J., D.L. and Y.C. provided patients and clinical data; D.Z. interpreted data, contributed to discussion, and reviewed the manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by the following funding agencies: National Natural Science Foundation of China (81572876, 81773087, 81071736 and 30973508 to H.Z.); Li Ka Shing Foundation Grant for Joint Research Program between Shantou University and Technion-Israel Institute of Technology, Shantou University, Guangdong Province.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.02.014.

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