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RAPID COMMUNICATION

Novel function of biguanides in inhibition of phospholipase D1 expression via a translational mechanism in cancer cells

Phospholipase D (PLD) and its product phosphatidic acid (PA) function as pleiotropic factors in the regulation of cancer progression, which includes the promotion of cell growth, survival, cell migration, and angiogenesis.[1] Chemical inhibition of the enzymatic activity of PLD1 and PLD2, or genetic down-regulation of their expression both induce tumor suppression,[1] while the compounds for PLD expression inhibition are barely reported. Here, we astonishingly found that the anti-hyperglycemic agents biguanides decreased PLD1 protein expression in cancer cells and further investigated its underlying regulatory mechanism.

Based on data from gene expression profiling interactive analysis (GEPIA), patients with elevated PLD1 expression have poorer survival in lung adenocarcinoma (LUAD), low-grade glioma (LGG), bladder urothelial carcinoma (BLCA), and stomach adenocarcinoma (STAD) (Fig. S1A–D). The representative cell lines of these above cancer types (A549, TCCSUP & EJ, HGC-27 are cell models for LUAD, BLCA, and STAD, respectively) and HeLa cells showed a nearly 30% reduction in the number of migrated cancer cells in both transwell and scratch-wound migration assays after PLD1 knockdown (Fig. S1E–G, S2A, B), while PLD1 overexpression promoted cell growth and migration in HeLa cells (Fig. S2F–H), suggesting the cancer-promoting function of PLD1 *in vitro*. Treatment of HeLa and A549 cells with metformin was shown to repress cell viability and cancer cell migration (Fig. S2C–E), while PLD1 overexpression negated the down-regulation of biguanides in cancer cell migration (Fig. S2G, H), which indicates that PLD1 might be a novel target of biguanides. As the elevation of PLD1 expression was reported in prostate cancer cells in comparison with normal prostate epithelium, and PLD1 expression is associated with the survival of cancer patients of LUAD, LGG, BLCA, and STAD, we studied the function of biguanides in the regulation of PLD1 and PLD2 expression in typical cell lines of BLCA, STAD, and prostate cancer cell line PC-3. The results showed that biguanides reduced PLD1 expression (Fig. S1H–K) in most of the above cell lines and this inhibition also existed in HeLa, A549, TR146, MCF-7, and HCT116 cells when metformin was present (Fig. 1A, B). Interestingly, different cancer cell lines responded differently to biguanide-induced PLD1 down-regulation. In contrast to TCCSUP, PLD1 expression remained unchanged in EJ cells after metformin or phenformin treatment although both are bladder cancer cell lines (Fig. S1H, I), and different responses to biguanides also exist in the other two cancer cell lines of STAD, HGC-27, and AGS (Fig. S1J; Fig. 1A, B). Surprisingly, the PLD2 protein expression was insensitive to either metformin or phenformin treatment (Fig. S1H–K; Fig. 1A) in most of the tested cancer cells. We further demonstrated that metformin down-regulated PLD1 protein expression in a time-dependent manner in both HeLa and A549 cells (Fig. 1C, D), and a low dose of metformin (40 μM) and phenformin (4 μM) that were closed to clinical plasma concentration of biguanides, also induced down-regulation of PLD1 protein expression for an elongated treatment time (Fig. 1E). In addition, we found that biguanide treatment did not affect the total PLD activities in the cells, whose levels were not significantly increased in both HeLa cells and PLD1-IRES-zsGreen overexpressed 293T cells (Fig. S2I, J), which suggested that the inhibitory roles of biguanides in cancer cell migration was independent on enzymatic regulation, but in down-regulation of PLD1 protein expression. We suspected that the unchanged total PLD activities in cells might be associated with other isoenzymes such as PLD2 because of its high basal activity and insensitivity to biguanides.

Biguanides have been shown to regulate several transcription factors, such as NF-κB, and influence endogenous protein expression. Our results showed that *PLD1* mRNA expression was mostly intact or even slightly increased in

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Abbreviations

4E-BP1	eukaryotic translation initiation factor 4E-binding protein 1
AMPK	AMP-activated protein kinase
ASNS	asparagine synthetase
BLCA	bladder urothelial carcinoma
CC	compound C
CHX	chlorhexidine
EIF4E	eukaryotic translation initiation factor 4E
EIF4G	eukaryotic translation initiation factor 4G
GEPIA	gene expression profiling interactive analysis
HIF1α	hypoxia-inducible factor 1-alpha
LGG	low-grade gliomas
LUAD	lung adenocarcinoma
Met	metformin
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa B
PA	phosphatidic acid
PABPs	poly-A binding proteins
Phe	phenformin
PLD	phospholipase D
PSAT1	phosphoserine aminotransferase 1
RNA-IP	RNA immunoprecipitation
PMA	phorbol myristate acetate
SK1	sphingosine kinase-1
STAD	stomach adenocarcinoma

the presence of biguanides (Fig. S3A bottom panel), whereas biguanide treatment diminished PLD1 protein expression in HeLa, A549, and TR146 cells (Fig. S3A upper panel). Metformin did not affect the dynamic expression of *PLD1* mRNA, whereas the *HIF1*α mRNA (positive control) was remarkably decreased after metformin treatment (Fig. S3B). Cotreatment of cycloheximide (CHX) with metformin did not accelerate the PLD1 degradation in both HeLa and TR146 cells (Fig. S3C, D). In addition, the treatment of neither ubiquitination inhibitor MG132 nor lysosomal degradation pathway inhibitor leupeptin was able to block the biguanide-induced down-regulation of PLD1 (Fig. S3E, F). Taken together, these above results suggested that both gene transcription and protein degradation pathways are not involved in the biguanide-induced down-regulation of PLD1 expression.

It has been reported that mutation-induced hyperactivation of the mTOR signaling pathway and selective translation of pro-survival and pro-invasion mRNAs are common in cancer cells.[2] As PLD1 acts as a tumorigenic factor and plays a key role in cancer cell survival and migration, we speculated that PLD might be one of these selective translation targets. As expected, the inhibitors (INK128 and Torin) of the mTOR signaling pathway decreased PLD1 expression (Fig. 1F, G). Moreover, compound C, an AMPK inhibitor, which has been shown to repress mTORC1 activation,[3] also inhibited PLD1 expression (Fig. 1G). Collectively, these results demonstrated that the mTOR signaling pathway is crucial for the regulation of PLD1 in cancer cells. Furthermore, we found that either knockdown of mTORC1 downstream effector 4E-BP1 or overexpression of EIF4E for promotion of the translation process increased PLD1 expression (Fig. 1H, I), which acted to abolish the function of biguanides on PLD1 (Fig. 1J, K), suggesting the involvement of translation modulation in biguanide-induced PLD1 down-regulation.

To directly test whether biguanides inhibit *PLD1* mRNA translation, we performed RNA-IP and subsequent RNA-seq experiments. We used an anti-EIF4E antibody to capture the EIF4E complex, which consists of mRNA, EIF4E, EIF4G, and poly-A binding proteins (PABPs). Immunoblotting experiments demonstrated that biguanide treatment reduced 4E-BP1 phosphorylation and increased the interaction of EIF4E and 4E-BP1 (Fig. 1L), suggesting the inhibition of mTORC1. We further used qPCR to measure the ratio of the captured mRNAs from RNA-IP and the total mRNAs harvested in cells. This ratio can be used to quantitatively analyze the translational efficiency of the target proteins. As expected, we found that the translational ratios of *PLD1* and *ASNS* (positive control) mRNA were greatly reduced in the presence of biguanides and CHX (Fig. 1M). Then, we generated a PLD1-IRES-zsGreen construct, where the sequences of *PLD1* and *zsGreen* were linked by the internal ribosome entry site (IRES) sequence. IRES sequence has been shown to recruit the ribosome in a cap-independent manner for the translation of subsequent mRNA sequences and has been widely used to produce individual proteins under the same promotor. The *PLD1* and *zsGreen* would be transcripted in a single mRNA molecular but translated into two individual proteins via IRES sequence. After low-dose metformin (40 μM) treatment, the EIF4G antibody was used to immunoprecipitate the translating mRNAs, and the translational ratios of *PLD1* and *zsGreen* mRNA were reduced synergistically (Fig. 1N, O). Additionally, we used a novel SunTag fluorescence imaging system to detect the translation process of *PLD1* mRNA in living cells.[4] The SunTag system consists of small SunTag peptides and a GFP-tagged single-chain variable fragment antibody (scFv-GFP) that can bind to the SunTag peptides such as GCN4 peptide, which allow us to detect the translating *PLD1* mRNA. The appearance of a single GFP cluster represents the translation of the *PLD1* mRNA, while the PP7-mChery probe was used to indicate the localization of *PLD1* mRNA. The GFP spots were decreased after phenformin treatment while the red signal remained unchanged (Fig. 1P, Q), suggesting an inhibition of the translation process of *PLD1* mRNA. Besides, RNA-seq results showed the translational ratios of many cancer and metabolism mRNAs also decreased after biguanide treatment (Fig. S4A–C). Sphingosine kinase-1 (SK1), an upstream regulator of PLD1, which has been previously identified as a target of biguanides.[5] Our results showed that the indirect activator of SK1, phorbol myristate acetate (PMA) partially rescued PLD1 expression after its cotreatment with metformin (Fig. S4D) in HeLa cells. Adversely, the SK1 inhibitor PF-543 decreased PLD1 expression in HeLa cells (Fig. S4E, F), and its cotreatment with biguanides induced more potent effects in the down-regulation of PLD1 expression (Fig. S4F right panel). Collectively, these results demonstrated that SK1 is partially involved in biguanide-induced PLD1 down-regulation.

To explore whether biguanides inhibit *PLD1* mRNA translation via AMPK activation, we investigated the expression of PLD after cotreatment of cancer cells with AMPK inhibitor compound C and metformin, and the action

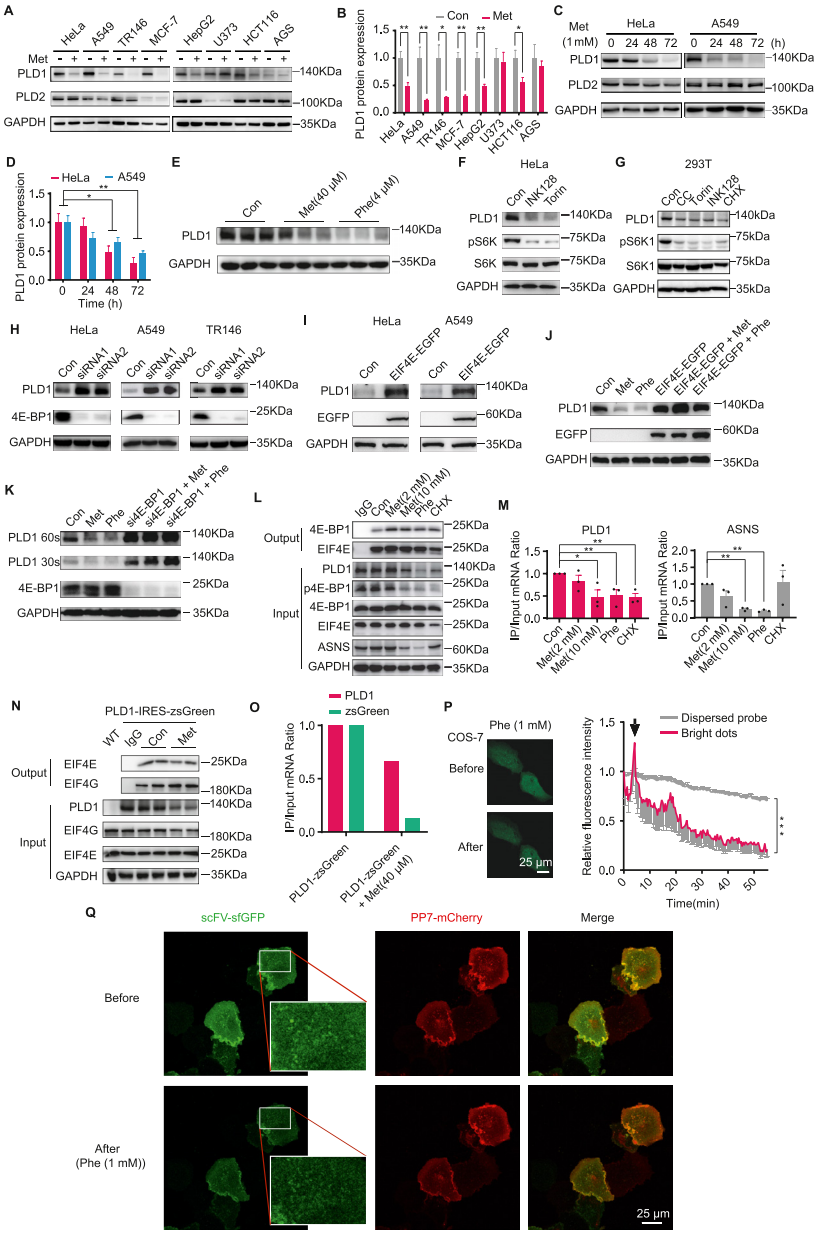

Figure 1 Biguanides inhibited PLD1 expression via a translational mechanism in cancer cells. **(A, B)** PLD1 and PLD2 expression in the indicated cancer cells after metformin (10 mM) treatment for 24 h. **(C, D)** The PLD1 and PLD2 expression in response to the low-dose metformin (1 mM) treatment for the indicated time. **(E)** The PLD1 expression after low-dose metformin (40 μM) and phenformin (4 μM) treatment in HeLa cells for five days. **(F)** The PLD1 protein expression in the presence of mTOR signaling pathway inhibitors for 24 h in HeLa cells. Con (DMSO), INK128 (500 nM), Torin (1 μM). **(G)** The PLD1 protein expression in the presence of mTOR signaling pathway inhibitor and protein biosynthesis inhibitor CHX for 48 h in 293T cells. Con (DMSO), Compound C (4 μM), Torin (1 μM), INK128 (100 nM), CHX (5 μM). **(H, I)** The PLD1 expression in the 4E-BP1-knockdown and EIF4E-overexpressed cancer cells. The EGFP antibody was used to detect EIF4E-EGFP expression. **(J, K)** The PLD1 expression in the 4E-BP1-knockdown and EIF4E-overexpressed HeLa cells after treatment with metformin (10 mM) and phenformin (500 μM) for 24 h. **(L)** The representative immunoblots of the RNA-IP assay. The EIF4E antibody was used to capture the binding mRNAs and ASNS was a positive target protein of biguanides, and CHX (10 μM) was the positive drug for translational inhibition. The duration of drug treatment was 24 h. **(M)** The translation ratios of PLD1 and ASNS in response to corresponding drug treatment for 24 h. **(N, O)** The representative immunoblots of the RNA-IP assay and the translation efficiency of *PLD1* and *zsGreen* mRNA with the EIF4G antibody after the clinical dose of metformin (40 μM) treatment for five days in the PLD1-IRES-zsGreen stably-expressing 293T cells. **(P)** Representative images and quantification that visualized the translation of PLD1 mRNA in live cells before and after phenformin (1 mM) treatment. The images were captured every 25 s for 60 min. Arrowhead indicates the time when phenformin was added. **(Q)** Visualization of the translation process of PLD1 mRNA in COS-7 cells before and after phenformin (1 mM) treatment for 60 min. The scFv-GFP probes dispersed in the cytoplasm would bind to the nascent peptide, which formed bright dots to visualize PLD1 translation. The PP7-mCherry probe was used to image the *PLD1* mRNA.

of biguanides on PLD1 expression in AMPK-knockdown cells. Astonishingly, we found that compound C did not rescue the biguanide-induced PLD1 down-regulation, and the inhibition of PLD1 expression by biguanides still existed even in AMPK-deficient cells (Fig. S4G, H). In summary, these results suggested that the biguanide-induced PLD1 down-regulation does not require AMPK activation.

In summary, our study illustrated that elevated PLD1 expression reduces cancer patients' survival, suggesting an important role of PLD1 in cancer progression. We found that biguanide treatment reduced PLD1 expression in cancer cells, and this down-regulation is not mediated by the gene transcription or protein degradation, nor dependent on AMPK activity. We further showed that biguanides selectively inhibited *PLD1* mRNA translational efficiency through the regulation of 4E-BP1 and EIF4E interaction. Although the inhibitory action of biguanides in PLD1 expression is not across all cancer cell lines and the mechanisms of the regulatory difference in different cell types remain unknown, our results suggest that PLD1 inhibition might be partially responsible for the preventative function of biguanides in cancer and biguanides might be beneficial to treat cancers with high PLD1 expression.

Author contributions

YX, YY, XW, and HL conceived and planned the experiments. YY, XW, XZ, YS, and WZ carried out the experiments. YX, JF, DK, and JZ supervised the project. YX and YY wrote the manuscript with inputs from all authors.

Conflict of interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.01.007.

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