Dihydropyrimidinase like 3 as a novel target of wild type p53 suppresses MAPK pathway in response to hypoxia

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Abstract: Endometrial cancer remains to be a major type of malignancy in threatening female life. Molecular insights in advancing our understanding of endometrial tumorigenesis are much needed. We here report that a less-studied protein Dihydropyrimidinase like 3 (DPYS3) is a potent tumor suppressor. DPYS3 is uniquely regulated by wild type p53 (wtp53), and its expression is at the highest level when cells carry wtp53 and are exposed to hypoxia. We reveal that wtp53 can bind DPYS3 promoter to enhance DPYS3 expression and in turn, the elevated DPYS3 can restrain cancer cell proliferation and invasion in vitro and in vivo. Importantly, we observe that DPYS3 can interfere with MAP kinase pathway, supported by a substantially reduced level of phosphorylated ERK in cells with high expression of DPYS3. Furthermore, we identify the specific region of DPYS3 that is responsible for its interaction with MEK and a subsequently reduced activity of ERK. In combination of molecular docking and mutagenesis analysis, we validated that the therapeutic implication of 17 A.A.s of DPYS3, which can reduce the activity of the MAPK pathway and inhibit endometrial tumor cell growth in vitro and in vivo. Therefore, our study not only demonstrates in-depth understanding of human tumorigenesis, especially endometrial tumor, but also only provides a therapeutic potential to develop an effective tool to fight against human malignancy.

Introduction

Endometrial cancer (EC) as one of the most common type of malignancy in female exhibits a rapid growth of morbidity around the world in recent years (Wright, 2016). EC can be broadly classified into estrogen dependent (Type I) and non-estrogen dependent (Type II) based on clinicopathologic feature and pathogenesis (Hafizz et al., 2020). More than 80% of newly diagnosed endometrial cancer cases in China are classified as Type I (Li et al., 2020). In the majority of type I cases, patients are often diagnosed at an early stage, and can be well healed under the standard surgical treatment and following chemotherapy (82% for 5-year disease free survival) (Bregar et al., 2017; Francis et al., 2019). However, those relapsed patients fail to get satisfactory therapeutic effect and show the poor prognosis due to malignant proliferation and distant metastasis (Connor and Rose, 2018; Polcher et al., 2019). To date, the underlying mechanism of endometrial cancer proliferation and distant metastasis remains uncertain.

Human solid tumors are invariably less well-oxygenated than the normal counterpart tissues from which they arose, albeit with variable incidence and severity within a given patient population. This so-called tumor hypoxia is a negative prognostic and predictive factor due to its resistance to radiotherapy and anticancer chemotherapy as well as predisposing for increased tumor metastases. Evidence from clinical and experimental studies increasingly point out that tumor hypoxia exert a variety of influences on activation of certain signal transduction pathways, gene regulatory mechanisms and induction of processes for DNA damage, tumor apoptosis and angiogenesis (Rankin and Giaccia, 2016; Wilson and Hay, 2011). The extent of hypoxia in these processes makes it an attractive therapeutic target for EC, and the novel hypoxia-regulated molecules that play a role in EC may provide more and better focus on therapeutic treatments.

Under conditions of hypoxia-induced stress, p53 is stabilized by the ATR and ATM kinases and facilitated by MDM2 reduction (Brosh and Rotter, 2009; Hammond et al., 2003) and leads to rapid apoptosis in oncogenically
transformed cells (Koumenis et al., 2001; Leszczynska et al., 2015; Sermeus and Michiels, 2011). However, merely hypoxia-induced p53 acts predominantly as a transrepressor rather than a transactivator during hypoxia (Hammond and Giaccia, 2005), which indicate that p53-dependent transactivation requires particular DNA damage induction, because long and severe hypoxia stress (<0.1% O2) has been proposed to be able to induce a replication stress-mediated DNA damage response and accumulates stabilization and activation of p53. Even so, the exact targets of p53-mediated transactivation responsible for apoptosis in hypoxia still remain poorly characterized (Johnson et al., 2005; Li et al., 2008).

Our previous RNA sequencing study revealed a novel gene, Dihydropyrimidinase like 3 (DPYSL3), highly expressed in tetracycline-induced p53 TET-on system in COLO-684 endometrial cancer cell line compared to un-induced control (data not shown). DPYSLs family (DPYSL1-5) is majorly highly expressed in nervous system and plays an essential role in neurite outgrowth, guidance and axonogenesis during neural development but generally decrease in adult brain (Charrier et al., 2003). Recent study reveals that DPYSL4 is a significant biomarker implicating with tumor malignancy in EC (Chen et al., 2015), indicating that DPYSL family may also participate in EC carcinogenesis. However, the function and mechanism of DPYSL3 are never illuminated. Now DPYSL3 is also observed highly expressed in COLO-684 cells with p53 over-expression. Therefore, we hypothesize that DPYSL3 might be a novel hypoxia-inducible target transactivator of p53. Our purpose in this study is to elucidate the involvement of DPYSL3 in p53 pathway and give understanding of its roles and mechanisms of action in human EC.

Materials and Methods

Cell lines, antibodies, chemicals, RNAi oligos and vector

All cell lines used in this study were obtained from Type Culture Collection of the Chinese Academy of Sciences (Beijing, China). Flag, DPYSL3, MEK, ERK, pERK and GAPDH primary antibodies were purchased from SAB Biotech (College Park, MD, USA). Deferoxamin and 5-FU were from Sigma-Aldrich (Billerica, MA, USA). DPYSL3 siRNA (5'-GGAUAUAACUCUACCACCAT-3') was synthesized from Geneharma, Shanghai, China. DPYSL3 cDNA was cloned into pIRE2-EGFP-SF plasmid (Vigene Biosciences, Jinan, China). The vectors of truncating DPYSL3 were prepared based on wild type DPYSL3 vector using mutagenesis PCR. The following PCR primer sequences were listed in Suppl. Table 1. The stably knockdown or knock-in DPYSL3 COLO-684, HEC-108, Ishikawa and HEC-59 cell lines were prepared via plasmid transfection using Lipofectamine 3000 (Thermo Scientific Fisher, Waltham, MA, USA), drug screening with 400 μg/mL G418 (Sigma-Aldrich) and maintained in DMEM (Gibco, Carlsbad, CA, USA) containing 10% (v/v) FBS (Gibco) supplemented with 250 μg/mL of G418.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed as previously described (Peng et al., 2020). Briefly, 5 × 10⁶ cells were fixed with 1% formaldehyde, quenched with 0.125 M glycine at room temperature and then lysed in 500 μL of lysis buffer (10 mM Tris-HCl (pH 8.0), 10 mM NaCl, and 0.2% IGEPAL CA-630 (Thermo Fisher Scientific)) on ice for 30 min. The genomic DNA was sonicated into 200–500 bp. Ten percent of each whole-cell lysate was stored as input, and the rest of the lysate was incubated with 1 μg appropriate antibodies at 4°C overnight. DNA fragments bound with protein were pull down by protein-A beads (Thermo Fisher Scientific) at 4°C for 2 h. Primers were designed to encompass about 100 bp up- and downstream of potential p53 DNA binding sites in the DPYSL3 gene. Their sequences were listed in Suppl. Table 1.

Reporter imaging assays

In brief, six genomic DNA fragments containing p53 binding sites of DPYSL3 (CBS1-4) were cloned into pGL3-promoter-reporter (Promega), respectively, and mixed with pcDNA3-wtp53 or mtp53 (R248G) as well as pRL-TK (Renilla luciferase-expressing construct; Promega), then cotransfected into Calu-6 cells. 48 h later, the Renilla-luciferase substrate coelenterazine (2.5 μg/mL) was added into the plates to detect the luciferase activity as a measure of transfection efficiency. The firefly luciferase substrate D-luciferin (100 μg/mL) was added for detection of p53-driven reporter activation.

Quantitative PCR (qPCR)

TRIzol (Thermo Fisher Scientific) was used to extract total RNA according to the manufacturer’s instructions. cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). All qPCR was performed using SYBR Premix Ex Taq Kit (Roche, Basel, Switzerland) to analysis mRNA. The primers were listed in Suppl. Table 1.

CCK-8 assay

1 × 10⁵ cells after 48-h transfection were plated in 96 well plate, followed by adding 10 μL CCK-8 solution (Solarbio, Beijing, China) to incubate additional 1 h. Absorption values of 450 nm were examined using Multiskan FC microplate reader (Thermo Fisher Scientific) to construct the regression equation and calculate IC50. The IC50 values were evaluated the cell proliferation.

Transwell assay

1 × 10⁵ cells were cultured within 200 μL suspension in upper and 800 μL fresh medium in lower transwell chamber (Corning, New York, NY, USA) on 24-well plates for 24 h. The cells at the lower chamber were cross-linked by 1% paraformaldehyde for 10 min and stained by 0.5% crystal violet (Sangon Biotech, Shanghai, China) for 5 min. The stained cells were counted under Olympus BX-51 microscope to evaluate the cell invasion.

Wound healing assay

1 × 10⁵ cells were sub-cultured in 6 well plate with 80% density, and a straight line was gently created using a p200 pipette tip. Cells were then washed with PBS and cultured in medium for the period of the assay. After 8-h and 24-h cell culture, the distance between the two sides of the scratch was captured, measured the dynamic change of width and
analyzed the cell migration by Image J. Amount of scratch closure was calculated as previously described (Ma et al., 2020).

**Immunoprecipitation and immunoblotting**

Transfected with appropriate plasmids and empty vectors used as the experimental controls accordingly, the whole cell lysates or nuclear extracts were mixed 1 μg Flag, MEK or Rabbit IgG antibody, and 40 μl flurry IgA beads (Invitrogen) for rotating overnight at 4°C. Immunoprecipitates were washed by IP buffer (20 mM HEPES [pH 7.9], 350 mM NaCl, 0.1% NP-40, 1 mM DTT, 0.2 mM PMSF, 2 mg/mL leupeptin and 2 mg/mL aprotinin) and western blotted for appropriate antibodies. For immunoblotting, the transfer-ready membranes were blocked overnight in TBS (10 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 5% nonfat milk and 0.1% Tween-20 at 4°C, followed by incubation with appropriate primary antibodies. The secondary antibodies of hors eradish peroxidase-conjugated anti-mouse, -rabbit, and -goat antibodies were used at a 1:5000 dilution.

**Xenograft mouse model**

Animal experiments in the current study were approved by our Institutional Laboratory Animals Committee and conducted following The Guidelines for Laboratory Animal Care and Use by the Affiliated Yantai Yuhuangding Hospital of Qingdao University. A total 16 BALB/c nude mice (male, weight 20–25 g) were supplied by Shanghai SLAC Laboratory Animal (Shanghai, China). Xenograft experiments were performed as described previously (Brown and Fan, 2016). In brief, mice were anesthetized using sodium pentobarbital (20 mg/kg). The stably DPYSL3 silencing COLO-684 cells and the stable DPYSL3 overexpressed Ishikawa cells (5 × 106) were suspended with 0.2 mL serum-free EGM-2 media and Matrigel matrix (v/v 1:1) and then injected onto the back of nude mice under sterile conditions. Tumor volume was calculated according to the formula: V = width 2 × length × 0.5. Mice were sacrificed on the 28th day.

**Protein docking**

The full length of amino acid of DPYSL3 and MEK were achieved and input into I-TASSER server (Zhang, 2008) (https://zhanglab.ccmb.med.umich.edu/) for homologous modeling by the comparison between candidate protein sequences and the known protein database, and given the protein structure. The models with highest score are selected. Both the files of DPYSL3 and MEK protein structure were put into Z-DOCK server (Pierce et al., 2014) (http://zdock.umassmed.edu/) to generate the protein docking. The given interaction complexes were visualized and analyzed by pyMOL to pick up confident model.

**Statistical analysis**

Data are presented as means ± standard deviations for three independent experiments. The difference of values was analyzed using Student’s t-test. P-values less than 0.05 was considered as statistical significance.

Commercialized kits, reagents, instruments, software, antibodies, etc., used in their research, shall be provided with their full name, along with the information of the Manufacturers/suppliers/software details (Name, City, Province/State, Country).

Accession numbers of RNA, DNA and protein sequences used in the manuscript should be provided.

**Results**

**Hypoxia stimulates DPYSL3 up-regulation in EC cell lines with wild type p53**

To validate the presence of DPYSL3 up-regulation by p53 over-expression in our previous RNA sequencing data, DPYSL3 mRNA and protein level were examined in EC cells of COLO-684 and HEC-108 (wild type p53, wtp53) as well as Ishikawa and HEC-59 (mutated p53, mtp53) treated with a hypoxia-mimic drug deferoxamine (DFO) or 5-fluorouracil (5-FU) known to damage DNA. The higher expression of DPYSL3 was observed after DFO or 5-FU exposure compared to non-treatment both in COLO-684 but not in Ishikawa cells (Figs. 1A–1D), which indicated that DPYSL3 was induced in a wild type p53-regulated manner under hypoxic stress and DNA damage condition.

**P53 is a binding target towards the promoter of DPYSL3**

As a transcription factor, p53 has been well revealed its DNA binding domain sequence (El-Deiry et al., 1992). Interestingly, DPYSL3 promoter region was also found containing four putative p53 consensus binding sequences (CBS). To verify the contribution of CBS1-4 of DPYSL3 to p53-dependent transcriptional regulation, all four CBSs were cloned individually and inserted into upstream of the minimal SV40 promoter in the pGL-3 luciferase reporter plasmid. CBS1, 2 and 4 reporters were significantly activated in COLO-684 and HEC-108 with wtp53. Notably, the activity of CBS1 and 4 were obviously compromised in Ishikawa (M246V) and HEC-59 (R273H) (Fig. 2A). To further determine the interaction between p53 and these candidate CBSs of DPYSL3, we conducted ChIP assay, and observed that CBS1, 2 and 4 were the preferential binding domains of wtp53 in COLO-684 and HEC-108 but showed lower binding ability by mtp53 in Ishikawa and HEC-59, while S3 could not be recognized by wtp53 or mtp53 (Fig. 2B). Taken together, our data demonstrated that p53 targeted DPYSL3 promoter region and facilitated its transcriptional level to respond the hypoxia stress.

**DPYSL3 inhibits tumor cells growth and invasion in vitro and in vivo**

Now that DPYSL3 has been demonstrated to be promoted by wtp53 under hypoxia condition in EC cells, therefore, the potential effect of DPYSL3 upon tumor cells were further investigated. Generating the stable DPYSL3 RNA interference EC cell lines COLO-684 and the stable DPYSL3 overexpression of Ishikawa (mtp53), we examined CCK-8 assay (Fig. 3A), transwell assay (Fig. 3B) and wound healing assay (Fig. 3C) and observed that DPYSL3 indeed inhibited cell growth and invasion. Furthermore, xenograft mouse models were also used to verify that tumor cells with overexpressed DPYSL3 grew slowly than control group, while silencing DPYSL3 enhanced the malignancy in vivo (Fig. 3D).
DPYSL3 interacts with MEK1 to suppress MAPK inactivation

To study the anti-proliferative mechanism of DPYSL3 in EC cells, we constructed Stag-Flag-DPYSL3 stably expressing COLO-684 cells, conducted Flag-IP and explored the probable binding protein of DPYSL3 using blue staining. Unlike in empty vector, one interested band near 45 kD was observed and identified as MEK1 using mass spectrometry assay. Moreover, to define the regions of DPYSL3 which are responsible for the interaction with MEK1, we performed a series of deletion analysis on DPYSL3 and conducted reverse IP to validate the interaction between DPYSL3 and MEK. We observed that the N-terminal (2–345) of DPYSL3 was essential for the interaction with MEK (Fig. 4A). And we further narrowed down the N-terminal of DPYSL3 and concluded that amino acids 2–16 and 143–159 (DPYSL3 tetramer interface domain) might be responsible for the interaction with MEK (Fig. 4B). Nevertheless, how does DPYSL3 affect MEK in tumor cells and whether MEK is involved in the inhibition of tumor cell proliferation mediated by DPYSL3 are both unknown. Therefore, we detected the phosphorylation level of ERK1/2 which is the downstream substrate of MEK1. Consistent with IP-assay, Thr202 phosphorylation level of ERK1/2 regulated by the DPYSL3 with A.A. 143–159 domain deletion group was

FIGURE 1. The expression of DPYSL3 in EC cell lines induced by hypoxia or DNA damage.

The mRNA and protein levels of DPYSL3 normalized by GAPDH in COLO-684 and HEC-108 (wild type p53) (A,B) as well as Ishikawa and HEC-59 (mutant p53) (C,D) before and after DFO or 5-FU treatment. Data are presented as the mean ± SEM of three individual experiments. * and # represent the comparison between DFO or 5-FU and control group with P < 0.05.

FIGURE 2. Interaction of p53 with the promoter of DPYSL3.

The luciferase activity derived from the promoter of DPYSL3 with different truncating p53 consensus binding sequences in EC cells (A). The enrichment of p53 on the promoter of DPYSL3 in EC cells (B). Data are presented as the mean ± SEM of three individual experiments. In panel A, * represents the comparison between CRS and blank groups, and # represents the comparison between Ishikawa/HEC-59 and COLO-684/HEC-108 groups with P < 0.05. In panel B, * represents the comparison between Ishikawa/HEC-59 and COLO-684/HEC-108 groups with P < 0.05.
higher compared to wild type and other DPYSL3 deletion groups (Fig. 4C), which indicated that the tetramer interface domain of DPYSL3 played an important role to suppress the MEK1 activity. Finally, we performed protein docking and speculated that the DPYSL3 might interact with MEK and suppress its kinase activity mainly through blocking MEK phosphorylation sites of 218 and 222 serine residues (Figs. 4D and 4E).

Discussion

DPYSL3 is an ectoderm specific gene, mainly expressed during nervous system development in newly born brain (Yasukawa et al., 2013), and silenced in mature neuron tissues. The presence of DPYSL3 high expression in EC cells promoted by wtp53 and hypoxia is firstly observed in our study (Fig. 1). In view of p53 high expression in early embryo or pluripotent stem cells (Solozobova and Blattner, 2011) and low expression in somatic normal tissues (Ashcroft and Vousden, 1999), as well as a partial hypoxia state of tightly controlled oxygen supply in early embryo, our results determine that DPYSL3 is comprehensibly regulated by both p53 and hypoxia stress in tumor cells. Moreover, given the less induction of DPYSL3 in the EC cells with mtp53 than wtp53 (Fig. 2), we speculate that first, hypoxia not p53 is essential to stimulate DPYSL3 transcription (Fig. 1B), secondly, p53 domains interacted with CBS motifs of DPYSL3 promoter region may not merely contain those variant mutant sites found in EC cells, and thirdly, these four CBS motifs may exert a different pattern and function for p53 promoting DPYSL3 transcription although CBS1, 2 and 4 are all observed binding with p53 from ChIP assay (Fig. 2D). Taken together, DPYSL3 is a novel downstream target gene of p53 upon the response of the hypoxia stress condition.

Recent studies revealed that the DPYSL family were highly expressed in breast and prostate cancers and negatively associated with invasiveness, metastasis, and motility (Cai et al., 2016; Casswell et al., 2015; Shih et al., 2003; Shih et al., 2001). Consistent with these previous
studies, our data suggested that DPYSL family may not only play a central role in nervous system development and pathology, but also a cancer suppressor gene that inhibits tumor cells proliferation and invasion (Fig. 3). Furthermore, the autoantibodies against DPYSLs observed in some non-neural neoplasm were able to cause severe neurological pathologies to those patients (Honnorat et al., 1999; Yu et al., 2001), which indicate that the abnormal DPYSL proteins, similar with p53, may become helpful for tumorigenesis.

The molecular function of DPYSL proteins mainly determine to be able to facilitate Sema3A-mediated morphological changes, such as microtubule dynamics regulation and cytoskeleton redistribution (Schmidt and Strittmatter, 2007). With the decipherment of DPYSLs crystal structure, the mechanism of DPYSLs regulating Sema3A/NP1/PlexA signaling pathway activation for neuron differentiation was primarily achieved via its N-terminal region and C-terminal (Deo et al., 2004). The N-terminal involved in constitutive activity termed “activation loop” is situated on the outer surface of DPYSLs monomer, allowing for regulation of other factors in the cytosol, while C-terminal contains the phosphorylation sites recognized by Cdk5, GSK3β is responsible for polymeric formation. In our study, the presence of both interaction with MEK1 and its kinase activity inhibition through the residues (amino acids 1-16 and 143-159) of DPYSL3 (Fig. 4B) provides an evidence that C-terminal of DPYSL3 is not required for MEK1 interaction and regulation, which implies that DPYSL3 may modulate MAPK pathway mainly via a monomer type in cancer cells. Given our protein docking model (Figs. 4D and 4E), the residues (amino acids 143-159) of DPYSL3 binding with the fraction including the phosphorylation sites of 218 and 222 serine residues of MEK1 suggest that DPYSL3 obstruct MEK1 phosphorylation sites to block MAPK pathway activation. Understanding DPYSL3 signaling provides significant clinical implications that DPYSL3 or its analogous peptide as the MAPK pathway inhibitor may be a novel therapeutic target for EC.

Conclusion

In this article, we suggest the role of DPYSL3 in the activity of MAPK/ERK pathway and the effects on tumor malignancy in EC. Our study provides a novel oncotarget for EC tumorigenesis and treatment.

Authors’ Contributions: DY and GW performed the experiments; LJ contributed to analyze the data and prepared the figures; ZR designed the overall project;
MJ drafted and revised the manuscript. The authors declare no competing financial interests exist.

Availability of Data and Materials: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPLEMENTARY TABLE 1

| Symbol name | Primer sequence |
|-------------|-----------------|
| DPYSL3 for qPCR | F: GGCGGAGATCCACGGTGGA  
R: GGGCCGTCATACAGTCCACCTG |
| GAPDH for qPCR | F: GAAGGTTGAAAGTCGGAGTC  
R: GAAGATGGTGATGGGATTTC |
| DPYSL3 CBS1 | F: ACTCTTCTTCTTCTACATAGCGCA  
R: GCTGATGCTGATCGGCGCAAC |
| DPYSL3 CBS2 | F: GGGGGAGCTGCTAGCTGTCTACA  
R: GTGTCAGTTGCTGATAC |
| DPYSL3 CBS3 | F: AATTTGAGCTGTAACAAAA  
R: TTAATAGCTTTACTCGATCACA |
| DPYSL3 CBS4 | F: AAATTCGTCGCACCTTAC  
R: CCGCGTCGGATGCTAAAAC |
| DPYSL3 full length | F: AATGTCTTTTCAGATTAAACTTA  
R: AATGCTGCTGCTGTACACAT |
| DPYSL3 2-16 deletion | F: GGATGACGATGACAAATACAAGG  
R: GCGTAAAAGGACTGGTCG |
| DPYSL3 17-345 deletion | F: TTCTTGCCAGTAGTGATG  
R: CTTATTACTGCTGCTCAGCAT |
| DPYSL3 346-551 deletion | F: CACCGCTGCTGATGCTGATGCTACAC  
R: CACGTGCTAGCTGCTAGTAC |
| DPYSL3 552-684 deletion | F: CCCGGCTGCTACTTCGTACACACAAA  
R: AATTITAGATTTTCTTATTATCTCAG |
| DPYSL3 17-142 deletion | F: CCGCGCTTCTTTCTGCTGCTCAGAACAAC  
R: CATTATGCTTTTCTGCTGCTACACAC |
| DPYSL3 143-159 deletion | F: ACACCTCTTTAGGCTGCTGATCGTAC  
R: ACACATGTGATCAGTCGGTTTACCAA |
| DPYSL3 160-245 deletion | F: CCCGGCTGCTTCTTTACTATTACCTCAGG  
R: GGGCTCGGTACCCGGATGCTTAC |

Note: All the primers used in this study are listed above.