International Journal of Pharmacology

ISSN 1811-7775

science alert

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OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2025.259.269

Research Article
PD-L1 Palmitoylation by ZDHHC3 Contributes to Chemoresistance and Inhibits CD8$^+$ T Cell Cytotoxicity in Gastric Cancer

Hui Li, Lin Zhu and Hongmin Wang

Medical School, Zhengzhou Sias University, Xinzheng, Henan, China

Abstract

Background and Objective: Programmed Death-Ligand 1 (PD-L1) is a crucial immune checkpoint in gastric cancer (GC) and its expression can be palmitoylated. However, the effect of PD-L1 palmitoylation in GC remains unclear. This study aimed to investigate the role of ZDHHC3-mediated PD-L1 palmitoylation in GC chemoresistance and CD8$^+$ T cell cytotoxicity. **Materials and Methods:** The expression levels of ZDHHC3 and PD-L1 in GC tissues were analyzed using public datasets. The interaction between ZDHHC3 and PD-L1 in GC cells (AGS) was investigated by co-immunoprecipitation and immunofluorescence. The effects of ZDHHC3 on PD-L1 stability, chemoresistance and CD8$^+$ T cell cytotoxicity were examined in AGS and AGS/DDP cells using siRNA knockdown and overexpression strategies. **Results:** The ZDHHC3 and PD-L1 were found to be coordinately overexpressed in GC and ZDHHC3 directly interacted with and stabilized PD-L1 in GC cells. Overexpression of ZDHHC3 conferred chemoresistance to GC cells through PD-L1, while ZDHHC3 knockdown sensitized GC cells to chemotherapy. Furthermore, ZDHHC3-mediated PD-L1 stabilization suppressed CD8$^+$ T cell cytotoxicity, which could be reversed by PD-L1 neutralization. **Conclusion:** The ZDHHC3-mediated PD-L1 palmitoylation contributes to chemoresistance and inhibits CD8$^+$ T cell cytotoxicity in GC. Targeting the ZDHHC3-PD-L1 axis may be a promising strategy to overcome chemoresistance and enhance anti-tumor immunity in GC.

Key words: Gastric cancer, programmed death-ligand 1, ZDHHC3, palmitoylation, chemoresistance

Citation: Li, H., L. Zhu and H. Wang, 2025. PD-L1 palmitoylation by ZDHHC3 contributes to chemoresistance and Inhibits CD8$^+$ T cell cytotoxicity in gastric cancer. Int. J. Pharmacol., 21: 259-269.

Corresponding Author: Hui Li, Medical School, Zhengzhou Sias University, No. 168, Renmin Road, Xinzheng 451100, Henan, China Tel: +86-15890167696

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Gastric cancer (GC) poses a significant global health challenge, being a highly prevalent malignancy and a primary contributor to cancer mortality rates worldwide[1]. Despite advances in diagnostic and therapeutic strategies including immunotherapy and precision medicine, gastric cancer patients continue to face a grim outlook, with only about one in five individuals surviving beyond five years post-diagnosis[2]. Since chemoresistance and immune evasion are two major obstacles in the treatment of GC[3], elucidating the intricate molecular pathways that drive these phenomena is a contributor to enhancing the outcome of affected individuals.

Programmed Death-Ligand 1 (PD-L1) is a crucial immune checkpoint protein that exerts an inhibitory effect on T cell activity through its interaction with the Programmed Death-1 (PD-1) receptor expressed on the surface of T lymphocytes, ultimately resulting in T cell exhaustion and immune suppression[4]. Moreover, PD-L1-induced upregulation of multidrug resistance genes can enhance drug efflux, leading to increased chemoresistance and survival of tumor cells[5]. This could be achieved through the activation of the complex signal pathways such as PI3K/AKT cascades, which regulate the expression of drug transporters[6]. These multiple roles of PD-L1 in immune evasion and chemoresistance highlight its importance as an interventional potential in cancer treatment. The PD-L1 overexpression was observed in patients with advanced-stage GC and correlated with diminished survival outcomes and reduced susceptibility to chemotherapeutic interventions[7-10]. Furthermore, knockdown of PD-L1 expression in human GC cells illustrated the efficacy of inhibiting tumor growth and progression, could enhance cell cycle arrest and increases its sensitivity to chemotherapeutic cytotoxicity[11]. However, the modulatory processes governing PD-L1 expression have not been fully elucidated.

Accumulating evidence suggests that PD-L1 expression is not only regulated at the transcriptional level but also subject to post-translational modifications (PTMs)[12]. Protein palmitoylation, a dynamic post-translational modification, involves the covalent attachment of a palmitate moiety to specific cysteine residues within target proteins mediated by ZDHHC protein acyltransferases[13]. Numerous studies have implicated that ZDHHC plays a significant role in the pathogenesis and advancement of several malignancies, such as those originating in the mammary glands[14], respiratory system and urinary system[15,16]. The inhibition of the DHHC3 enzyme impairs the palmitoylation of PD-L1, leading to its endocytic recycling and subsequent lysosomal degradation. This mechanism has been shown to exert a suppressive effect on MC38 tumors in mice, demonstrating the importance of ZDHHC's regulation on PD-L1 in the intricate molecular landscape of cancer[17]. However, the specific involvement of these modifications in the context of GC has yet to be thoroughly explored and warrants further investigation.

The present study investigated the role of ZDHHC3-mediated PD-L1 palmitoylation in GC progression and underlying mechanisms. The findings demonstrated the correlation between ZDHHC3 and PD-L1 in GC cell lines and that ZDHHC3 overexpression conferred chemoresistance to GC cells and suppressed $CD8^+$ T cell-mediated cytotoxicity through stabilizing PD-L1, which highlights the ZDHHC3/PD-L1 axis as a promising interventional candidate for GC treatment.

MATERIALS AND METHODS

Study area: The present study was performed at Zhengzhou Sias University from May, 2022 to October, 2023.

Bioinformatic analysis: The expression profiles of ZDHHC3 and CD274 (PD-L1) in stomach adenocarcinoma (STAD) were obtained from the online database Gene Expression Profiling Interactive Analysis[18]. The correlation between ZDHHC3 and CD274 expression in STAD was investigated using the online database The Encyclopedia of RNA Interactomes (ENCORI) and further analyzed using STAD data from the BioXpress Database (BD)[19,20]. The Pearson correlation coefficient (r) and p-value were calculated to determine the relationship between ZDHHC3 and CD274 expression in STAD.

Cell lines and culture: The human gastric cancer cell line AGS was obtained from the Procell Life Science and Technology Co., Ltd. (CL-0022; Wuhan, China). Cells were cultured in RPMI-1640 medium (Gibco, Waltham, Massachusetts, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The cisplatin (DDP)-resistant AGS/DDP cell line was established by continuously exposing parental AGS cells to increasing concentrations of DDP (Sigma-Aldrich, St. Louis, Missouri, USA)[21]. The maintained AGS cells were treated with 0.5 μM cisplatin for 72 hrs and the surviving cells were cultured and exposed to DDP incremented by 0.5 μM every two weeks until a final concentration of 2 μM was reached. Then, cells were maintained in an RPMI-1640 medium containing 2 μM cisplatin to preserve the drug-resistant phenotype.

Plasmids and small interfering RNA transfection: The ZDHHC3 or PD-L1 overexpression plasmid and siRNAs targeting ZDHHC3 or PD-L1 were designed, synthesized and confirmed by GenePharma (Shanghai, China). Plasmids, siRNAs and their corresponding negative controls were either individually or co-transfected into AGS or AGS/DDP cells using Lipofectamine 3000 (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. At 48 hrs post-transfection, the expression level of the target protein was verified by the western blot to confirm the transfection efficiency.

Quantitative Real-Time PCR (qRT-PCR): Total RNA was extracted from tissues using TRIzol reagent (invitrogen) and reverse-transcribed into cDNA using the PrimeScript RT Reagent kit (Takara, Kusatsu, Japan). The qRT-PCR was performed using the SYBR Premix Ex Taq II kit (Takara) on a LightCycler 480 system (Roche, Mannheim, Germany). The PCR conditions were set as follows initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 9°C for 5 sec and annealing and extension at 60°C for 30 sec. The primer sequences were listed in Table 1. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the internal control.

Co-Immunoprecipitation (Co-IP): The Co-IP was performed to investigate the interaction between ZDHHC3 and PD-L1. The AGS cells were lysed in ice-cold RIPA buffer (Roche) to obtain total protein samples, whose concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA) according to the manufacturer's instructions. The total protein samples were pre-cleared by incubation with control IgG antibody (Cell Signaling Technology, Danvers, Massachusetts, USA) and Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Dallas, Texas, USA) for 1 hr at 4°C and then were incubated with anti-ZDHHC3 antibody or specific anti-PD-L1 antibody overnight at 4°C. The next day, beads were added and incubated for 2 hrs at 4°C to capture the antibody-antigen complexes. The immunoprecipitates were washed five times with cold RIPA buffer and then eluted with 2×SDS-PAGE sample buffer. Subsequently, the eluted protein samples were subjected to SDS-PAGE electrophoresis for western blot analysis.

Cycloheximide (CHX) assay: The AGS cells with or without ZDHHC3 knockdown were seeded in 6-well plates at a density of 2×10^5 cells per well and cultured until the cells reached 70-80% confluence. They were then treated with 50 μg/mL CHX (Sigma-Aldrich) to inhibit protein synthesis. Cells were harvested at 1, 2, 4, 6 and 8 hrs post-CHX treatment using RIPA lysis buffer and PD-L1 protein expression was evaluated by the western blot.

Western blot: Treated AGS cells were lysed in RIPA buffer. Equal amounts of proteins were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Burlington, Massachusetts, USA). The membranes were blocked with 5% skim milk and then incubated with primary antibodies against ZDHHC3, PD-L1 and GAPDH (Table 2, diluted at 1:500 for use) overnight at 4°C. After washing with Tris-Buffered Saline with Tween 20, the membranes were incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 hr at room temperature. The protein bands were visualized using an enhanced chemiluminescence kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) and imaged using a ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, California, USA). The band intensities were quantified using ImageJ version 1.34 software (National Institutes of Health, Bethesda, Maryland, USA) and normalized to the corresponding loading control GAPDH.

Immunofluorescence (IF): After 48 hrs of transfection with vector control or ZDHHC3 overexpression plasmid, the AGS cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min and blocked with 5% bovine serum albumin for 1 hr. The cells were then incubated with primary antibody against PD-L1 (1:200, Table 2) overnight at 4°C, followed by incubation with Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody (1:500; Invitrogen) for 1 hr at room temperature. Nuclei were counterstained with DAPI for 5 min. The coverslips were mounted and IF images were acquired using a Zeiss LSM 880 confocal microscope (Carl Zeiss, Oberkochen, Germany). The fluorescence intensity of PD-L1 was quantified using ImageJ version 1.34 software.

Table 1: Primer sequences used in this study

Gene	Sequence	
DHHC3	Forward	GGCCCTGCTCTTCCTCATTT
	Reverse	CACTTGACGTACCAAGCCCT
CD274	Forward	TGCTGCCCTTCAGATCACAG
	Reverse	GGGCATTGACTTTCAGCGTG
GAPDH	Forward	GGCAAATTCAACGGCACAGT
	Reverse	GGCCTCACCCCATTTGATGT
si-ZDHHC3-1		AGUACUUCUGGUUGUUCUCGC
si-ZDHHC3-2		UUUUUUAGCCCAUCUUCUCUC

Table 2: Antibodies used in this research

Antibody	Manufacturer	Cat.no
ZDHHC3	Bioss Inc.	bs-24751R
PD-L1	BosterBio	M00109-3
GAPDH	BosterBio	M00227-7

Cell viability determination: The AGS cells transfected with either empty vector or ZDHHC3 overexpression plasmid and AGS/DDP with either si-NC or si-ZDHHC3 RNAs were treated with various concentrations of DDP (0, 0.5, 1, 2, 5, 10, to 20 μM) for an additional 24 hrs. Cell viability was then assessed using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. Briefly, 10 μL of CCK-8 solution was added to each well of cells and the plates were incubated for 2 hrs at 37°C. The absorbance at 450 nm was measured using a microplate reader (BioTek Instruments, Winooski, Vermont, USA).

Colony formation assays: The AGS cells transfected with either empty vector (Vector), ZDHHC3 overexpression plasmid (ZDHHC3), si-PD-L1 or a combination of ZDHHC3 overexpression plasmid and si-PD-L1 (ZDHHC3+si-PD-L1) were treated with 1 μM DDP, while AGS/DDP cells transfected with either si-NC, si-ZDHHC3, PD-L1 overexpression plasmid (PD-L1) or a combination of si-ZDHHC3 and PD-L1 overexpression plasmid (si-ZDHHC3+PD-L1) were treated with 2 μM DDP. The cells were then seeded in 6-well plates at a density of 1×10^3 cells for 14-day culture. After incubation, the colonies were fixed and stained with crystal violet. The colonies were counted using a microscope (Olympus SZX16, Olympus Corporation, Tokyo, Japan).

$CD8^+$ T cell-mediated cytotoxicity assay: Human peripheral blood mononuclear cells were isolated from blood derived from healthy donors using the Lymphoprep (10970, Stemcell Technologies, Vancouver, Canada) according to the manufacturer's protocol. To obtain activated T cells, PBMCs were cultured in CTS™ AIM V™ SFM medium (A3021002, Gibco) supplemented with ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator (10970; STEMCELL Technologies) and recombinant human IL-2 (1000 U/mL; 11848-HNAY1, Sino Biological, Beijing, China) for 1 week[22,23]. The AGS cells with ZDHHC3 knockdown were seeded in 24-well plates and cultured alone or co-cultured with activated T cells for 48 hrs with a ratio of 1:3. After 48 hrs incubation, the surviving cancer cells in the wells were washed with PBS and then fixed with 4% paraformaldehyde for 15 min. The 0.1% crystal violet solution was used for staining and was subsequently solubilized using 10% acetic acid. The optical density was measured at 570 nm. To investigate whether the role of ZDHHC3 in immune escape is mediated by PD-L1, a PD-L1 neutralizing antibody (10084-R639, Sino Biological) was added to the co-culture system in the ZDHHC3 overexpression group at a concentration of 10 μg/mL. The surviving cancer cells post 48 hrs incubation were quantified as described above.

Statistical analysis: Statistical analyses were performed using GraphPad Prism software (version 8.0; GraphPad Software, San Diego, California, USA). All data were presented as Mean±Standard Error of the Mean (SEM). For comparisons among groups, One-way Analysis of Variance (ANOVA) followed by Tukey's *post hoc* test was conducted for normally distributed data, whereas Kruskal-Wallis test followed by Dunn's *post hoc* test was used for non-normally distributed data. Correlation analyses were performed using Pearson's correlation coefficient. A two-tailed p-value less than 0.05 was considered statistically significant.

RESULTS

ZDHHC3 and PD-L1 are coordinately overexpressed in STAD tissues: To determine the relevance of ZDHHC3 and PD-L1 in GC, their mRNA expression levels were first analyzed based on publicly available datasets. Analysis of the data derived from GEPIA revealed that both ZDHHC3 and CD274 were significantly increased in STAD tissues than those in SHPP tissues (Fig. 1a-b). Moreover, correlation analysis using the ENCORI and BD databases showed a positive correlation between ZDHHC3 and CD274 levels in STAD samples (Fig. 1c). These data suggest that ZDHHC3 and PD-L1 are coordinately over expressed in STAD, implying a potential regulatory relationship between them.

ZDHHC3 stabilizes PD-L1 in GC cells through direct interaction: The functional interaction between ZDHHC3 and PD-L1 *in vitro* using the human GC cell line AGS was subsequently explored. Over-expression of ZDHHC3 in AGS cells significantly increased PD-L1 protein levels, as determined by western blot (Fig. 2a) and IF analyses (Fig. 2b). Conversely, knockdown of ZDHHC3 using both independent siRNAs led to a marked reduction in PD-L1 protein expression (Fig. 2c) and fluorescence performance expression (Fig. 2d). To test the interaction of ZDHHC3 with PD-L1, we performed co-IP, which demonstrated a direct interaction between endogenous DHHC3 and PD-L1 in AGS cells (Fig. 2e). Furthermore, CHX assay revealed that DHHC3 knockdown accelerated the degradation of PD-L1 protein, with its protein level significantly lower than that of the si-NC group starting from the 4th h (Fig. 2f). This suggested that ZDHHC3 could stabilize PD-L1 in GC cells.

ZDHHC3 confers chemoresistance to GC cells through PD-L1: To investigate the role of ZDHHC3 in GC chemoresistance, we established a AGS/DDP cell line by continuous exposure to increasing concentrations of DDP. After treatment of DDP, AGS

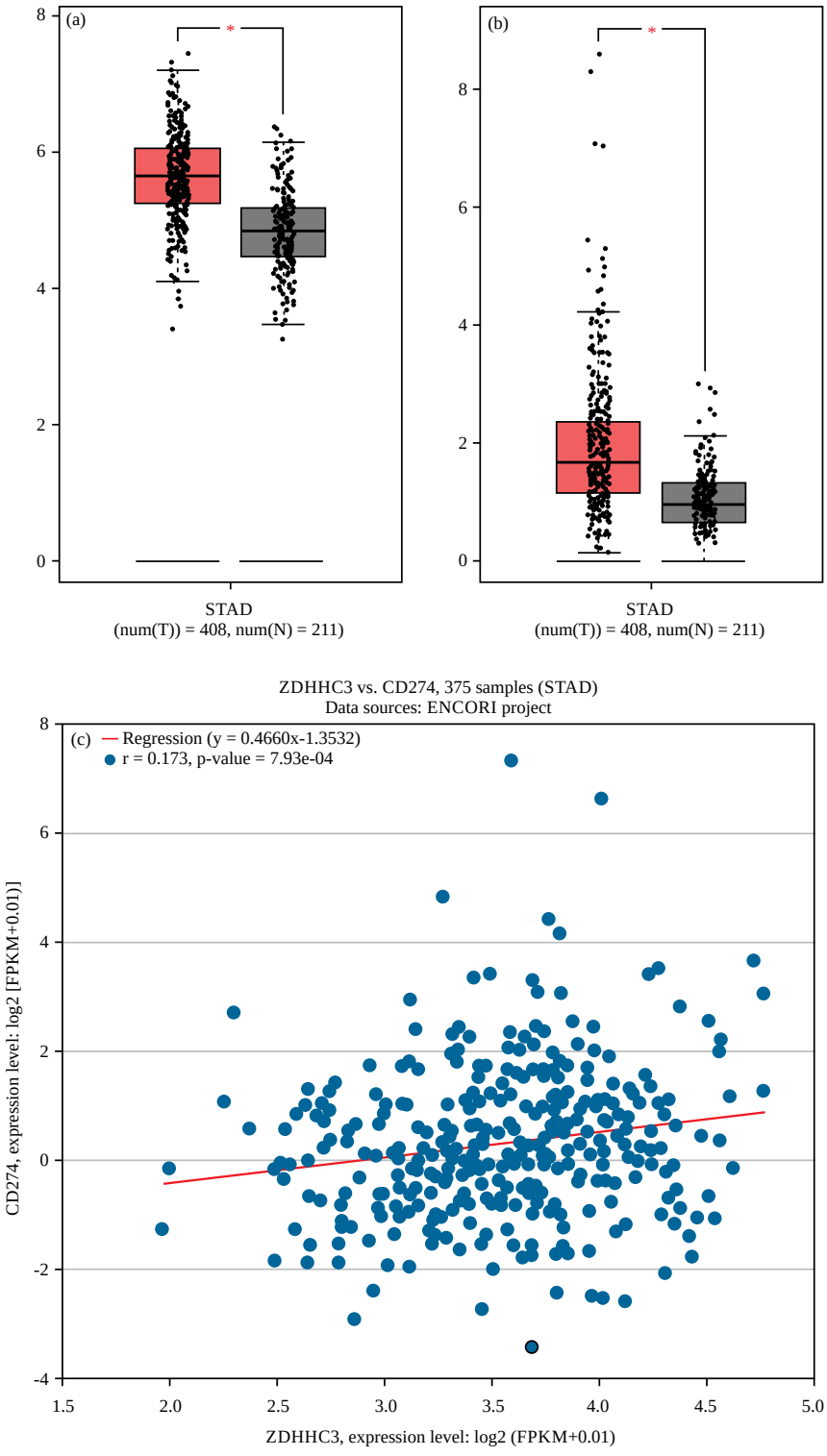

Fig. 1(a-c): ZDHHC3 and CD274 were highly expressed and exhibited a positive correlation in STAD tissues, (a) ZDHHC3 and (b) CD274 expression levels in STAD tissues were analyzed using data derived from the Gene Expression Profiling Interactive Analysis database, Correlation analysis between ZDHHC3 and CD274 expression in STAD samples using the (c) Encyclopedia of RNA Interactomes

Data are presented as Mean±SEM, *p<0.05, **p<0.01 and STAD: Stomach adenocarcinoma

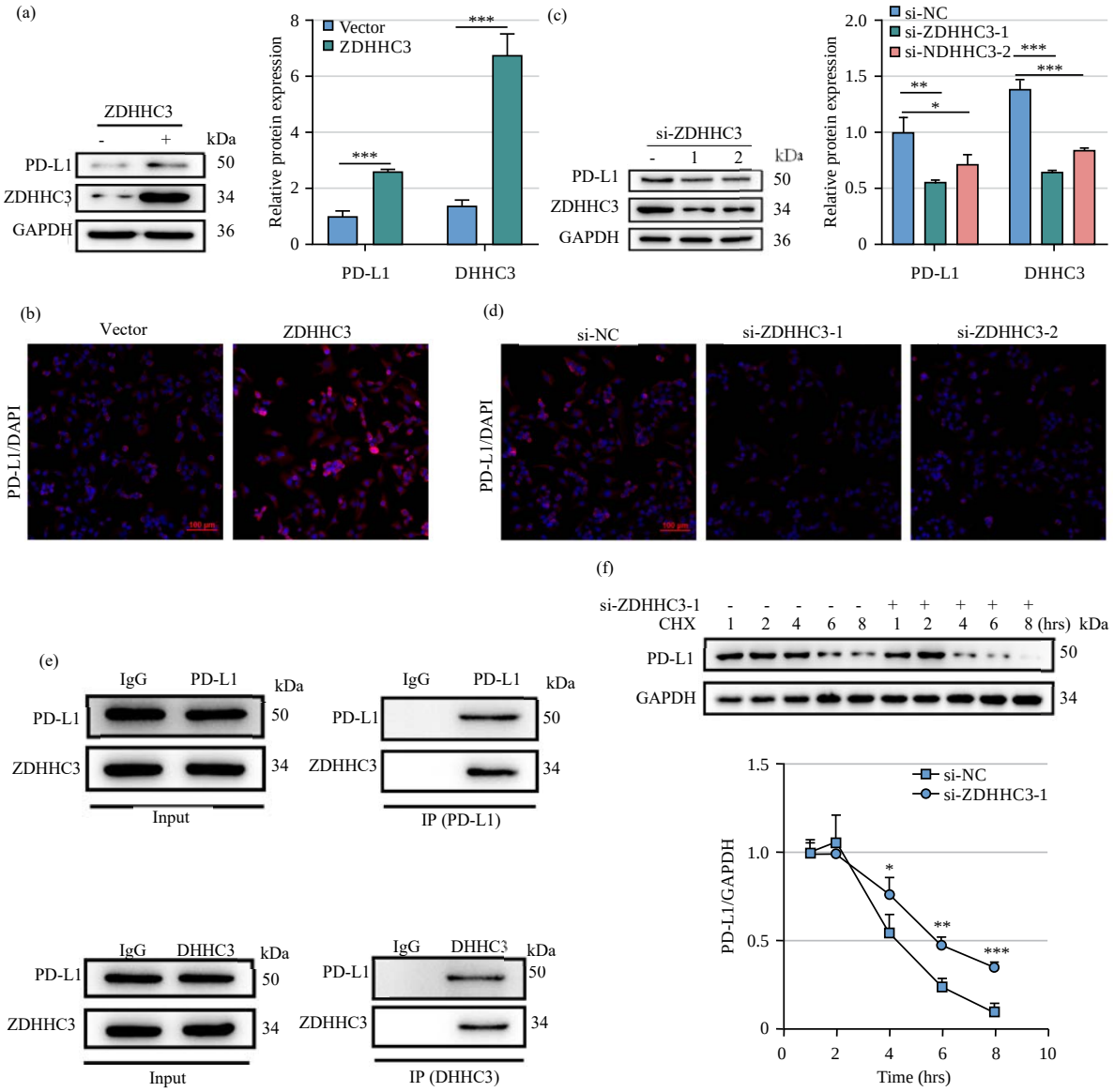

Fig. 2(a-f): ZDHHC3 acetyltransferase increased PD-L1 stabilization in gastric cancer cells, (a) Western blot analysis of PD-L1 and ZDHH3 protein levels in AGS cells overexpressing ZDHHC3, (b) Immunofluorescence analysis of PD-L1 expression in AGS cells overexpressing ZDHHC3, (c) Western blot analysis of PD-L1 and ZDHH3 protein levels in AGS cells transfected with two independent siRNAs targeting ZDHHC3, (d) Fluorescence performance expression of PD-L1 after transfection, (e) Co-immunoprecipitation analysis of the interaction between endogenous ZDHHC3 and PD-L1 and (f) Cycloheximide assay to determine the degradation rate of PD-L1 protein in ZDHHC3 knockdown AGS cells transfected with siRNA or negative control at h 1, 2, 4, 6 and 8 hrs after treatment

Data are presented as Mean±SEM, *p<0.05, **p<0.01 and ***p<0.001

with transfection of ZDHHC3 showed significantly higher IC_{50} value of DDP compared to the control group (Fig. 3a-b), whereas, ZDHHC3 knockdown sensitized AGS/DDP cells to DDP and reduced IC_{50} value (Fig. 3c-d). After 1 μM DDP treatment, colony formation assay suggested that the faster AGS cell proliferation conferred by ZDHHC3 overexpression could be reversed by PD-L1 knockdown (Fig. 3d). Consistently, ZDHHC3 knockdown inhibited proliferation of AGS/DDP cell under 2 μM DDP treatment, which was partly abrogated by introduction of PD-L1 (Fig. 3e). These findings indicated that ZDHHC3 played a crucial role in the chemoresistance of gastric cancer cells by regulating PD-L1 expression.

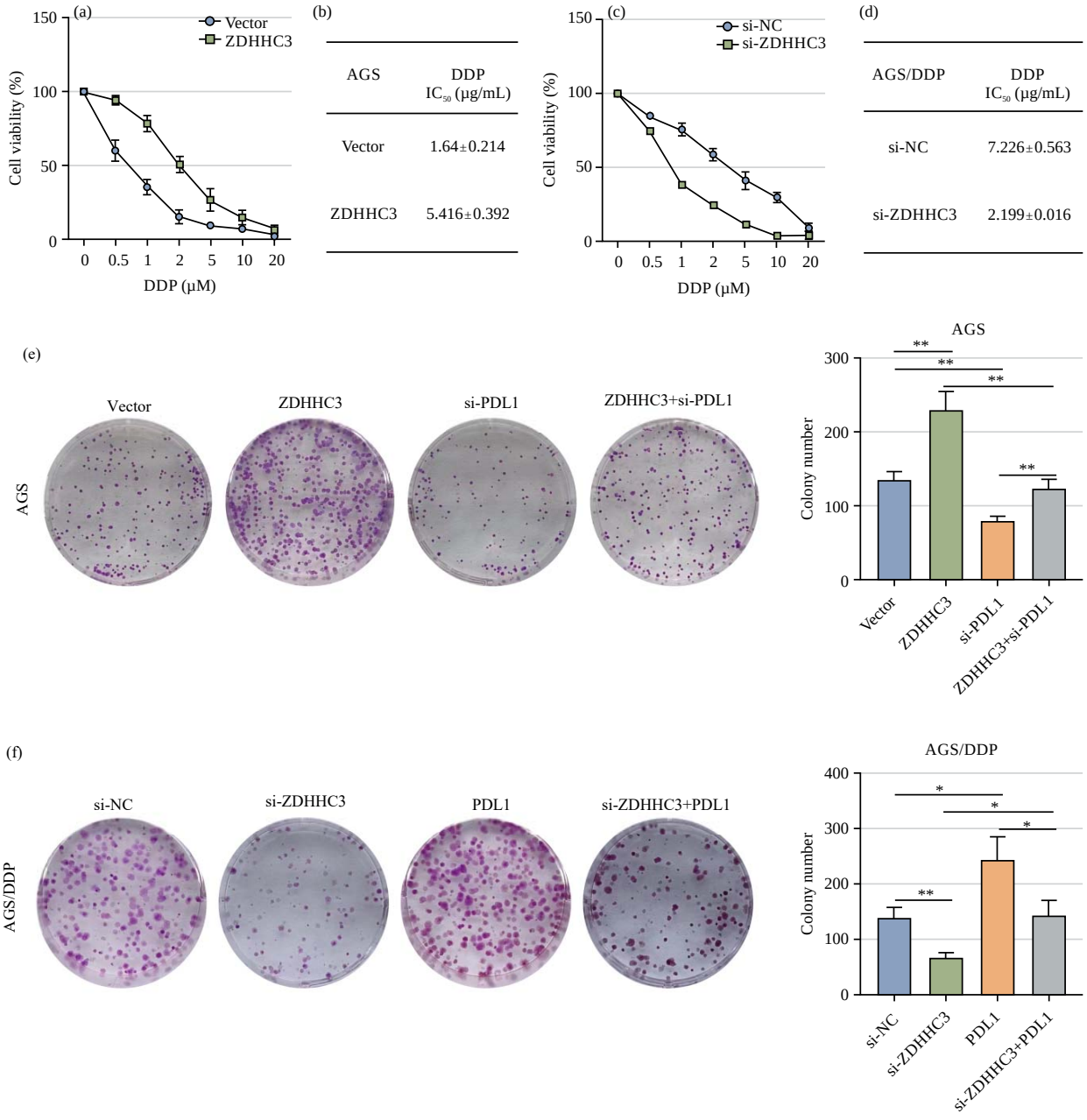

Fig. 3(a-f): DHHC3 conferred chemoresistance of DDP to GC cells by PD-L1, (a-b) IC_{50} values of DDP in AGS cells transfected with ZDHHC3 overexpression plasmid or control vector were determined by cell viability assay, (c-d) IC_{50} values of DDP in AGS/DDP cells transfected with ZDHHC3 siRNA or negative control si-NC were determined by cell viability assay, (e) Colony formation assay of AGS cells overexpressing ZDHHC3 with or without PD-L1 knockdown under 1 μM DDP treatment and (f) AGS/DDP cells transfected with ZDHHC3 siRNA with or without PD-L1 overexpression under 2 μM DDP treatment

Data are presented as Mean±SEM, *p<0.05, **p<0.01 and DDP: Cisplatin

ZDHHC3 suppresses $CD8^+$ T cell-mediated cytotoxicity through enhancing PD-L1 expression: To determine whether ZDHHC3-mediated PD-L1 stabilization affects T cell function, we co-cultured AGS cells with activated T cells derived from healthy donors at a 1:3 ratio. After 48 hrs incubation, knockdown of ZDHHC3 significantly promoted $CD8^+$ T cell-mediated killing of AGS cells, as evidenced by the higher survival of AGS cells with si-NC treatment than those

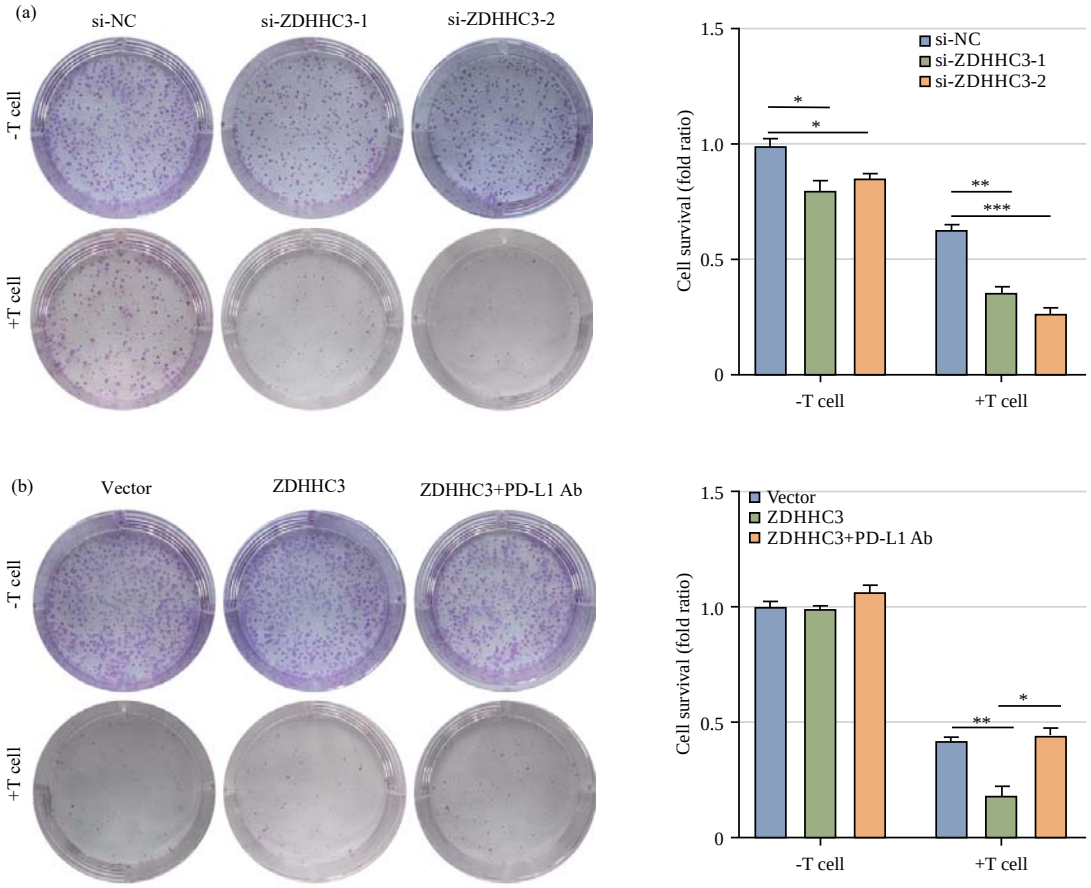

Fig. 4(a-b): ZDHHC3 overexpression inhibits CD8⁺ T cell cytotoxicity to GC cells. Activated T cells were obtained by culturing PBMCs from healthy donors with human CD3/CD28/CD2 T cell activator and recombinant human IL-2 for one week, (a) si-ZDHHC3 or si-NC treated AGS cells were cultured with or without activated T cells at a 1:3 ratio for 48 hrs. The survival of AGS cells was assessed by crystal violet staining and (b) AGS cells overexpressing ZDHHC3 were co-cultured with activated T cells in the presence or absence of a blocking antibody against PD-L1, survived tumor cells was stained by crystal violet after 48 hrs of co-culture

Data are presented as Mean±SEM, *p<0.05, **p<0.01, ***p<0.001 and PBMCs: Peripheral Blood Mononuclear Cells

with si-ZDHHC3 (Fig. 4a). Moreover, the increased tumor cell viability induced by ZDHHC3 overexpression was reversed by the blocking antibody against PD-L1, indicating that ZDHHC3 inhibits CD8⁺ T cell cytotoxicity through upregulating PD-L1 (Fig. 4b).

DISCUSSION

Based on bioinformatic analysis combined with *in vitro* experiment, the present study demonstrated that ZDHHC3 conferred chemoresistance to GC cells and suppresses CD8⁺ T cell-mediated cytotoxicity through stabilizing PD-L1, suggesting its potential to be considered as a strategy for suppressing GC progression and mitigating immune evasion. The GC poses a substantial burden on healthcare systems, with its elevated occurrence and significant contribution to cancer-related deaths[24]. Due to its suboptimal prognosis under current management regimen exploring novel therapeutic targets is beneficial for improving the outcomes of patients[25].

As a widely recognized immune checkpoint, PD-L1-targeted therapies have shown promising results in various cancers including GC. In the clinical trial, pembrolizumab monotherapy showed an ORR of 11.6% in PD-L1-positive advanced GC patients, whereas a PD-1 inhibitor, nivolumab, demonstrated an ORR of 11.2% in them[26,27]. However, not all patients respond to these therapies and resistance can develop over time[28]. To understand the regulating principles underlying PD-L1 expression to improve the efficacy of existing PD-L1-targeted therapies, it is illustrated that PD-L1 palmitoylation is present and plays a functional role in our preliminary investigation.

Protein palmitoylation could regulate proteins encoded by oncogenes and tumor suppressors[29]. The overexpression of ZDHHC3 was found as a crucial marker in various cancer types[30-33]. In line with the current study findings, in which the increased ZDHHC3 mRNA levels were observed in STAD patients through both online databases and our human samples. Importantly, the simultaneous upregulation and positive correlation between ZDHHC3 and PD-L1 suggest their association in GC progression, which was directly confirmed by subsequent Co-IP. Through this direct interaction, PD-L1 is shielded from degradation, reflected in the finding that addition of CHX to ZDHHC3-knockdown AGS cells resulted in accelerated PD-L1 degradation. This is consistent with previous studies describing that ZDHHC3-mediated palmitoylation of PD-L1 at cysteine residue 272 protects PD-L1 from degradation by inhibiting its ubiquitination[34,35].

In functional investigations, this stabilizing effect of ZDHHC3 on PD-L1 might contribute to the chemoresistance of GC tumor cells and their evasion of $CD8^+$ T cell-mediated cytotoxicity, which remains two major obstacles in improving GC patient outcomes and overall survival rates[36]. Tumor cells can develop resistance to chemotherapy through several mechanisms related to PD-L1, like that PD-L1 could promote the migratory and invasive properties of sorafenib-resistant hepatocellular carcinoma cells, human GC tissues and DDP-resistant GC cells via the PI3K/AKT pathway[37]. Additionally, the suppressive effect of miR-34a-5p on PD-L1 expression was found to attenuate the resistance of DDP-refractory ovarian cancer cells to the chemotherapeutic agent, cisplatin DDP[38]. This trend was also illustrated in current study, in which the suppression of AGS cell proliferation by ZDHHC3 overexpression under DDP treatment could be reversed through PD-L1 knockdown. Conversely, the chemoresistance effect limited by ZDHHC3 knockdown on AGS/DDP cells exhibited an increasing trend upon the addition of PD-L1, suggesting that the modulation of ZDHHC3 can influence the chemoresistance of GC cells mediated by the PD-L1 level modulation.

Furthermore, the stabilization of PD-L1 by ZDHHC3-mediated palmitoylation can inhibit $CD8^+$ T cell cytotoxicity. These cytotoxic T lymphocytes have the ability to identify and eradicate malignant cells through a complex process involving the targeted secretion of specialized granules[39]. Immune checkpoint blockade therapies targeting the PD-1/PD-L1 pathway have shown remarkable success in restoring the anti-tumor function of $CD8^+$ T cells in various cancer types[40]. For example, nivolumab that selectively blocks the interaction between PD-1 and its ligands were illustrated the capability of restoring the anti-tumor activity of T cells and demonstrated promising efficacy in clinical trials involving GC patients[41]. In present study, the addition of a PD-L1 neutralizing antibody similarly demonstrated a significant reduction in the survival of ZDHHC3-overexpressing GC cells when co-cultured with T cells. The restoration of $CD8^+$ T cell function upon PD-L1 blockade highlights the promising role of the ZDHHC3/PD-L1 axis in regulating anti-tumor immunity.

To validate the generalizability of the current study findings, future studies should investigate the ZDHHC3-PD-L1 axis in additional GC cell lines with varying molecular and phenotypic characteristics instead of AGS alone. Moreover, *in vivo* models should be exploited in further studies, such as patient-derived xenograft or syngeneic mouse models, to validate the role of the PD-L1 palmitoylation in regulating chemoresistance and anti-tumor immunity in a more physiologically relevant context to provide more reliable evidence.

CONCLUSION

This study demonstrated that ZDHHC3 and PD-L1 are coordinately overexpressed in STAD tissues and ZDHHC3 directly interacts with and stabilizes PD-L1 in GC cells. Furthermore, ZDHHC3 not only confers chemoresistance to GC cells under DDP treatment through PD-L1 but also suppresses $CD8^+$ T cell-mediated cytotoxicity mediated by PD-L1 stabilization, which can be reversed by PD-L1 blockade. Taken together, current findings highlight the crucial role of ZDHHC3-mediated PD-L1 palmitoylation in promoting chemoresistance and inhibiting anti-tumor immunity in GC. Targeting the ZDHHC3-PD-L1 axis may provide a novel therapeutic strategy to improve the efficacy of immunotherapy and optimize patient outcomes.

SIGNIFICANCE STATEMENT

The present study elucidated the role of ZDHHC3 in regulating PD-L1 expression and ZDHHC3-mediated PD-L1 palmitoylation in gastric cancer (GC). By demonstrating the coordinated overexpression of ZDHHC3 and PD-L1 in gastric cancer tissues and the direct interaction between these two proteins, present work provides insights into the molecular mechanisms underlying PD-L1 stability and its contribution to chemoresistance and immune evasion. The findings suggested that targeting the ZDHHC3-PD-L1 axis may represent a promising therapeutic strategy for overcoming chemoresistance and enhancing anti-tumor immunity in GC patients and lays the foundation for further exploration of ZDHHC3 as a potential biomarker and therapeutic target in GC, with implications for improving patient outcomes and overall survival rates.

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