EJGO European Journal of Gynaecological Oncology

GCDH contributes to better outcome and acts on chemoresistance and immune exclusion in cervical cancer

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Summary

Aim: Cervical cancer is a major health problem in women and its genetic culprit remains inclusive. The authors aim to evaluate the role of glutaryl-CoA dehydrogenase (GCDH) in cervical cancer. *Materials and Methods:* Clinicopathological attribution of GCDH was queried in silico using the human cancer genome atlas project (TCGA) and HPA database. In vitro studies using cell lines and nude mice were used to examine the therapeutic potential of targeting GCDH. *Results:* GCDH expression was significantly higher in cancer tissue compared with normal cervical tissue. Cervical cancer patients with overexpressed GCDH had significantly better overall survival. Functional annotation indicated enrichment in cell-cycle regulatory pathway. Knockdown (KD) and overexpression (OE) of GCDH-induced increased and decreased proliferation, respectively. Similar results were also obtained in cell-cycle arrest, invasion, migration, and colony formation assays, but having no effect on cell apoptosis. To understand the selection advantage of GCHD-overexpressed cases, the authors analyzed immunological profile in silico. Expression of GCDH correlated with that of IDO2, which was significantly associated with decreased immunity. GCDH-KD-induced decreased expression of IDO2, which also led to decreased kynurenine and tryptophan in culturing media. GCDH-OE promoted chemoresistance in cervical cancer cells due to cell-cycle arrest. GCDH-OE also significantly inhibited tumor growth in vivo yet showed partial resistance to chemotherapy, with increased level of IDO2 expression. *Conclusion:* GCDH is overexpressed in cervical cancer and represents a less aggressive phenotype of disease, which is also characterized with increased chemoresistance and immune exclusion. Inhibition of IDO2 could be of therapeutic potential.

Key words: Cervical cancer; GCDH; Chemoresistance; Immune exclusion.

Introduction

Cervical cancer is the fourth most common cancer worldwide and is the fourth leading cause of death in female cancer patient. Incidence and mortality of cervical cancer constitutes 8%, respectively, among all cancers [1]. About 70% of cervical cancer occur in developing countries and remains the most common cause of death in low-income countries [2]. HPV is closely associated with cervical cancer. Among the variety of HPV subtypes, type 16 and 18 are associated with substantially higher susceptibility. Though vaccination can now block these two types at almost 100% success rate, only 65-70% of cervical cancer can be attributed to HPV 16 or 18, leaving ~40% of patients unprotected [3]. In the USA, there is an overall five-year survival of 68% and early detection is of great importance for the outcome as a precocious surgical intervention offers excellent tumor control. Given the lack of obvious symptoms, absence of routine smear test or timely vaccination, which is common in less developed countries, may finally lead to diagnosis at a late stage, where a combination of surgery, chemo- or radiotherapy only provides marginal effects [4]. Novel treatment modalities are an urgent need.

The human cancer genome atlas project (TCGA) has revealed a genetic landscape of a variety of cancers including cervical cancer. Many genetic culprits that play a role in carcinogenesis not revealed before can now be not only detected but also targeted [5]. Glutaryl-CoA dehydrogenase (GCDH) is a gene located on chromosome 19 and it catalyzes the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA and carbon dioxide in the degradative pathway of L-lysine, L-hydroxylysine, and L-tryptophan metabolism [6]. Mutation in GCDH leads to the formation and accumulation of the metabolites glutaric acid and 3-hydroxyglutaric acid, as well as glutarylcarnitine in body fluids, which essentially leads to glutaric aciduria type I, an autosomal recessive metabolic disorder [7]. The role of GCDH in cancer remains elusive. To date, very few studies reported the role of GCDH in any type of cancer, let alone in cervical cancer. One study reported in limited cases that GCDH mutation was noted in gastric and colorectal cancers, yet no mechanistic analysis was performed [8].

In the current study, the authors have for the first time analyzed role of GCDH in cervical cancer. They have in part revealed the mechanism of selective advantage favoring GCDH-overexpressed cervical cancer that is characterized with a better outcome. Detailed regulatory mechanism

Revised manuscript accepted for publication October 29, 2018

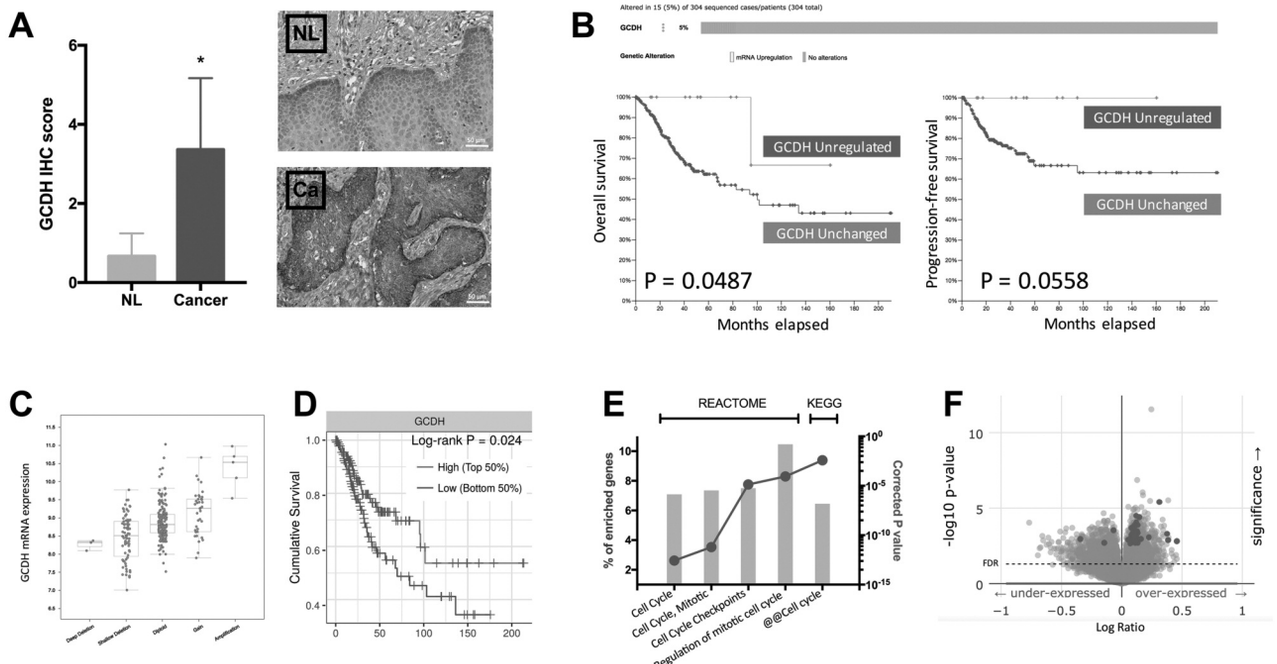

Figure 1. — Expression of GCDH in cervical cancer. A) GCDH expression in healthy cervical tissue and cervical cancer, reproduced from human protein atlas. Reproduced from TCGA dataset, are B) expression of GCDH in patient samples of cervical cancer and relevant survival profile, C) GCDH expression in relation to copy number of the gene, D) overall survival when grouped by top *vs.* bottom 50% of GCDH expression level, E) functional annotation of genes enriched in GCDH-overexpressed cases, and F) volcano plots showing highlighted (red dots) cell cycle related genes enriched in GCDH-overexpressed cases (*$p < 0.05$, **$p < 0.01$).

between GCDH and immune modulation or cell cycle mediation warrants further investigation.

Materials And Methods

The cervical cancer subset (CSEA) of TCGA was reproduced to study expression of GCDH in cervical cancer using the cBioPortal platform [5, 9, 10], which contained RNA seq data of 309 cervical cancer samples. Clinicopathological information including age, tumor stage and grade, survival period, and follow-up time was extracted. Expression status of GCDH was shown using the OncoPrint function of cBioPortal online. List of genes coexpressed with GCDH detected using RNA seq was generated using the coexpression function of cBioPortal online, and the Pearson test was opted for correlation evaluation. Genes passing the ±40 of coefficient R were input to the KOBAS 3.0 platform for functional annotation [11, 12]. Only KEGG Pathway and Reactome datasets were allowed for annotation.

Expression of GCDH in normal and cancerous kidney tissue was evaluated semi-quantitatively using the Human Protein Atlas platform [13-16]. The captured normal cervix tissue was via the following link (https://www.proteinatlas.org/ENSG00000105607-GCDH/tissue/cervix%2C+uterine). Cropped representative images for cervical cancer can be accessed via https://www.proteinatlas.org/ENSG00000105607-GCDH/pathology/tissue/cervical+cancer. The extensity and intensity of staining was graded as follows as per established protocol [17, 18], namely: 1 for light yellow, 2 for dark yellow, and 3 for brown. Sum of extensity and intensity represents the final quantification of each sample: 0 for negative (1-2), 1 for mild (3), 2 for moderate (4), and 3 for strong (5-6).

Immune estimates were profiled using an online analytical tool named "TIMER". TIMER is a web resource for systematical evaluations of the clinical impact of different immune cells in diverse cancer types [19]. The immune estimate of TCGA-CSEA dataset was obtained and plotted from TIMER platform. The Gene module on TIMER provided the correlation of selected gene expression with various immune infiltration level. The Survival module illustrated the Kaplan-Meier curve and provided the Cox regression analysis between overall survival and clinical factors, immune infiltrates or gene expression level.

The Genomics of Drug Sensitivity in Cancer (GDSC) dataset was used to profile the inhibitory concentration (IC50) of cisplatin in cervical cancer cell lines used in this study. IC50 of cisplatin across multiple cervical cancer cell lines were plotted and cut-off between sensitivity and resistance were automatically generated online.

Human HeLa and SiHa cervical cancer cells were obtained . Cells were cultured in RPMI 1640 with 10% fetal bovine serum. Cisplatin was added once at indicated does (6.9 μM for HeLa and 16.5 μM for SiHa). Proliferation was examined using crystal violet (CV) assay, which was applied at each time point (0, 24, 48, and 72 hours). After the medium was gently removed, cells were fixed with 10% formalin for five minutes and were then stained with 0.05% CV for 30 minutes. Straight methanol was then applied and plates were read at absorbance of OD 540 nm.

Lentivirus encoding GCDH was generated by the full-length ORF. Cells were plated in 12-well plates transduced with 5 MOI lentiviral particles, and incubated at 37°C, 5% CO_2. Overexpression of GCDH in stable transfected cells was confirmed by qPCR. shRNA constructs were obtained from The RNAi Consortium (TRC, http://www.broadinstitute.org/rnai/public/). Procedures for vector transduction was well established [20]. Two transcripts were chosen (TRCN0000275836 and TRCN0000220897) and transduction was performed in both cervical cell lines. Representative data were pre-

Table 1. — *Association between GCDH expression and clinicopathological parameters.*

				GCDH expression		
		n	%	Mean	SD	P value
Stage						
	I	162	52.4	-0.11	1.983	0.975
	II	70	22.7	-0.18	2.166	
	III	46	14.9	-0.02	2.214	
	IV	21	6.8	-0.21	0.818	
	N/A	10	3.2	/	/	
Metastasis						
	No	115	37.2	-0.16	2.082	0.676
	Yes	10	3.2	-0.46	2.96	
	N/A	184	59.5	/	/	
Grade						
	1	18	5.8	-0.89	2.786	< 0.001
	2	136	44	0.07	1.613	
	3	119	38.5	-0.12	2.149	
	4	1	0.3	-6.85	/	
	N/A	35	11.3	/	/	
Pathology						
	Adenosquamous carcinoma	5	1.6	-0.03	0.944	0.889
	Endometrioid carcinoma	3	1	0.08	0.67	
	Mucinous carcinoma	17	5.5	-0.33	1.559	
	Cervical	2	0.6	-0.37	0.094	
	Squamous cell carcinoma	255	82.5	-0.14	1.973	
	Endocervical adenocarcinoma	27	8.7	0.34	2.462	
BMI						
	Underweight	11	3.6	-0.42	0.796	0.464
	Normal	87	28.2	0.12	1.878	
	Overweight	75	24.3	-0.35	2.127	
	Obese	88	28.5	-0.05	1.998	
	N/A	48	15.5			
		Mean	SD	r		P value
Lymph node involvement		1.05	2.40	0.022		0.781
Age		48.26	13.81	-0.019		0.738
BMI		28.11	7.65	0.009		0.886

N/A = not accessible; SD = standard deviation.

sented with HeLa cells.

Total RNA of was extracted with Trizol. After concentration was determined, RNAs were converted to cDNAs. Forward and reverse primers of GCDH and internal control GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were GCDH F 5'-CGT CCC GAG TTT GAC TGG C-3' and R 5'-GAT GCG AGG CAT GAG TCT CT-3'; GAPDH F 5'- ATG GGG AAG GTG AAG GTC G-3', and R 5'-GGG GTC ATT GAT GGC AAC AAT A-3'. For each sample, the average value of threshold cycle was normalized to GAPDH level with the formula, $2^{-\Delta\Delta Ct}$.

Total protein was extracted and concentration was determined by BCA assay. Electrophoresis separation on SDS-PAGE gel was performed and proteins were transferred to PVDF membrane using at constant voltage. After blockade overnight, primary antibodies was applied for incubation (anti-GCDH at 1:500, anti-IDO1 at 1:100, anti-IDO2 at 1:100, anti-GAPDH at 1:1000) for 90 minutes at room temperature and HRP labelled rabbit-anti-mouse secondary antibody was applied. Protein was detected using enhanced chemiluminescence and autoradiography.

The cell cycle and apoptosis were measured using flow cytometry. For cell cycle analysis, cells were trypsinized and treated with cell cycle staining buffer for 15 minutes. Suspension were then subject to flow cytometry on a flow cytometer. For apoptosis, cells were stained with Annexin V-fluorescein and propidium iodide (PI) (BD) for 15 minutes at room temperature. Samples were then analyzed with flow cytometry to determine percentages of apoptotic cells using Annexin V/PI indication.

Cells were seeded in the top chamber of the 8.0-μm pore size cell culture inserts that were either coated or uncoated with matrigel for migration and invasion assays,, respectively. Inserts were placed in a 24-well plate filled with complete medium. Cells that penetrated to the underside surfaces of the inserts were fixed and stained with the CV method and were counted under the microscope. Assay was done in triplicates. Colony formation was performed using soft agar assay. Briefly, 1,000 cells were resuspended in complete medium mixed with 0.4 % agarose, which was layered on the top of 0.6% agar in medium supplemented with 20% FBS on 60-mm plates. After two weeks of culture at 37°C, plates were stained with 0.005% of crystal violet for one hour. Colonies were counted microscopically and the relative colony numbers were measured.

Forty-eight male BALB/c nude mice at six weeks of age were bred in special pathogen-free (SPF) grade laboratory. Mice were randomly divided into eight groups. A total of 10^7 cancer cells resuspended in 100 ml of PBS were injected subcutaneously at the left axilla of each mouse. Tumors became perceptible at approximately 5 mm in diameter on approximately day 7. For the cisplatin-treatment group, low-dose regime was given (0.5 mg/kg i.p

Table 2. — *Cox-regression model demonstrating impact of GCDH on survival.*

	B	SE	Wald	df	*p* value	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Grade			1.853	3	0.603			
G1	7.199	49.326	0.021	1	0.884	1338.349	0	1.30E+45
G2	8.108	49.322	0.027	1	0.869	3321.276	0	3.20E+45
G3	7.901	49.322	0.026	1	0.873	2699.953	0	2.60E+45
GCDH	-0.137	0.058	5.594	1	0.018	0.872	0.778	0.977

SE = standard error ; CI = confidence interval.

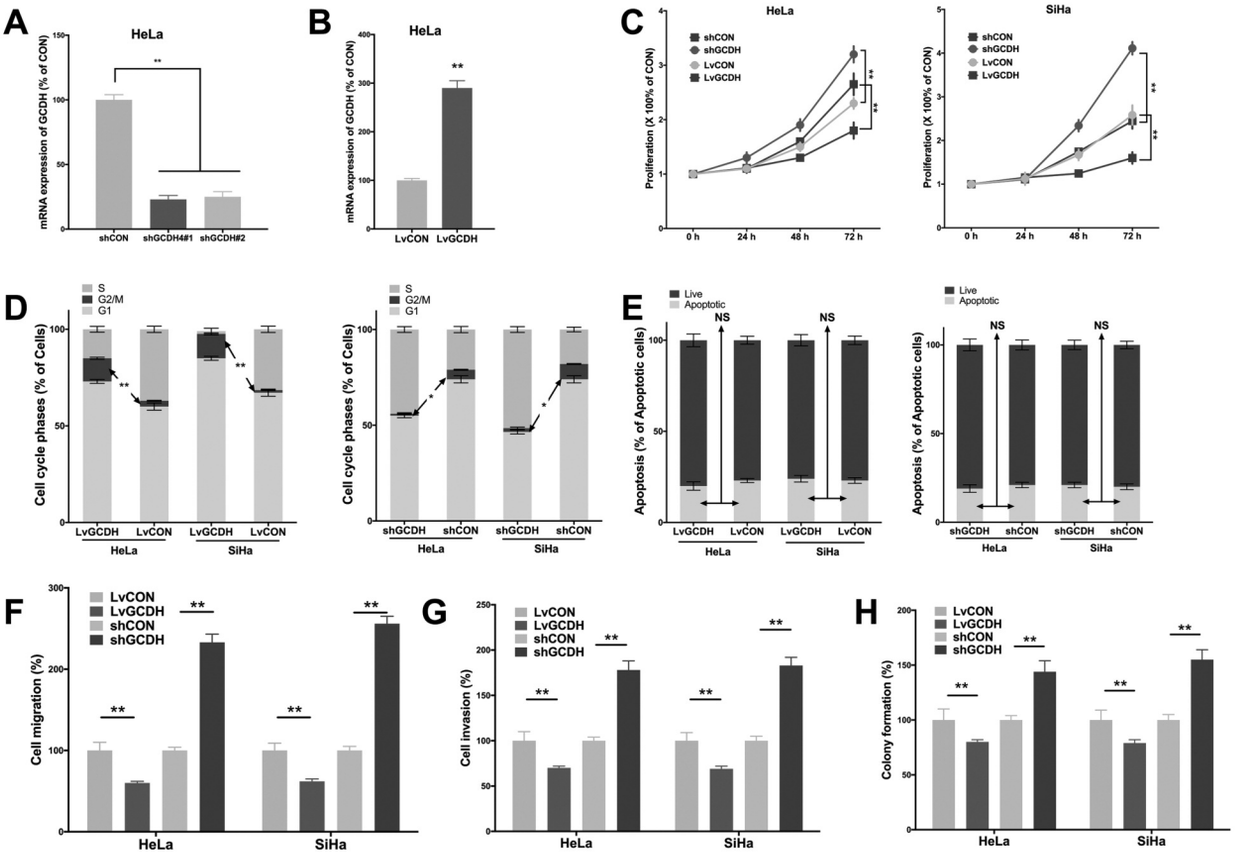

Figure 2. — GCDH overexpression inhibits proliferation of cervical cancer cell lines. Efficacy of A) knockdown by shRNA and B) overexpression by lentiviral vector in HeLa cells; comparisons of C) proliferation, D) cell cycle profile, E) cell apoptosis, F) migration, G) Invasion, and H) colony formation between cells with different genetic status of GCDH in both cervical cancer cell lines (*$p < 0.05$, **$p < 0.01$).

for seven days). All mice were sacrificed on Day 35 and tumors were extracted. Tumor size was calculated with the formula, volume = length×width^2/2.

Comparisons between the two groups were analyzed with the Student's *t*-test. For clinicopathological parameters, unevenly distributed parameters in the univariate analysis were included in the multivariate analysis, for which the Cox regression model was used. The *p* value of less than 0.05 was accepted as statistical significance.

Results

The authors first studied GCDH expression in healthy and cancerous tissue of uterine cervix evaluated with IHC score and found that GCDH expression was significantly higher in cervical cancer (Figure 1A). Among cervical cancer cases, overexpression of GCDH was present in 5% of cases, that demonstrated significantly prolonged overall survival and a trend towards longer progression-free survival (Figure 1B). Although the latter did not reach statistical significance, there was not a single case relapsed in the GCDH-upregulated cohort (Figure 1B). The authors then analyzed expression level of GCDH related to copy number of the gene and found that expression was higher with copy number amplification, indicating a functioning gene product in cervical cancer (Figure 1C). The authors thus further grouped cases by

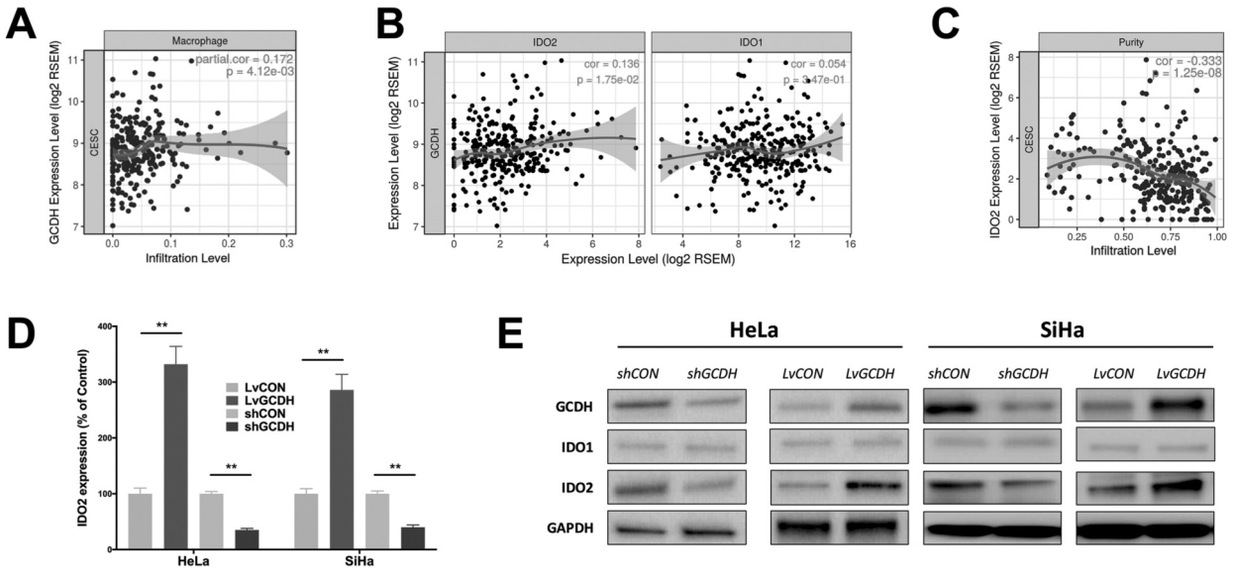

Figure 3. — GCDH and immunity in cervical cancer. Reproduced from TCGA dataset using TIMER program; shown are A) relation between GCDH expression and tumor-infiltrating macrophage, B) relation between GCDH expression and expressions of IDO1 and IDO2, C) relation between IDO2 expression and tumor-infiltrating lymphocytes, D) expression of IDO2 in cervical cancer cells with different GCDH status, and E) alteration of IDO1 and IDO2 when GCDH was genetically altered in cervical cancer cell lines (*$p <$ 0.05, **$p < 0.01$).

top versus bottom 50% of GCDH expression and found that cases with higher GCDH expression had significantly longer overall survival (Figure 1D). To further rule out confounders, they listed clinico-pathological parameters between cases with upregulated and unchanged cases and found the only unevenly distributed factor was tumor grade (Table 1). To examine whether GCDH-related better outcome was due to lower tumor graded associated thereto, the authors included both factors into the multivariate regression model and found that GCDH was an independent favorable factor for cervical cancer (Table 2). A functional probing was then carried out by targeting gene expression enrichment between cases with upregulated and unchanged GSDH. Functional annotation of enriched genes pointed towards cell cycle control from both REACTOME and KEGG datasets (Figure 1E). The volcano plot revealed that upregulation of several genes that could induce cell cycle arrest in GCDH-overexpressed cases (Figure 1F). Here the authors demonstrated GCDH was upregulated in cervical cancer and was associated with better outcome. Its upregulation could be related to cell cycle arrest.

To further understand the findings, the authors carried out in vitro assay in two cervical cancer cell lines. They first tested efficacy of KD and OE in the cells (Figures 2A-B). As expected, GCDH-OE significantly inhibited cell proliferation, whereas GCDH-KD significantly increased cell proliferation in both cell lines (Figure 2C). Of note, GCDH-OE induced significant cell population in G2/M phase, whereas GCDH-KD induced significantly less population in both cell lines (Figure 2D). Interesting, neither OE or KD of GCDH had any effect on cell apoptosis in either cell line (Figure 2C). GCDH-OE also significantly inhibited cell migration and invasion, whereas GCDH-KD significantly increased cell migration and invasion in both cell lines (Figures 2D-E). Similar results were also observed in the colony formation assay that profiled anchorage-independent growth (Figure 2F). Here the authors have validated regulatory role GCDH in cell cycle arrest in vitro. Halted cell cycle progression but not apoptosis could be one of the main mechanisms through which the proliferation, invasion, and colony formation were decreased in case of GCDH-OE.

As GCDH was closely related to tryptophan metabolism, the authors then investigated the role of GCDH in immune modulation. Indoleamine-pyrrole 2,3-dioxygenase genes (IDO1 and IDO2) played a key role in the kynurenine pathway, mediating O_2-dependent oxidation of L-tryptophan to N-formylkynurenine, which mediated immune tolerance in a variety of cancers. The authors first observed a positive correlation between GCDH expression and tumor-infiltrating macrophages (Figure 3A). A positive correlation was demonstrated between expressions of GCDH and IDO2 but not IDO1 (Figure 3B). Overexpression of IDO2 was significantly associated with less tumor-infiltrating lymphocytes (TILs) in cervical cancer (Figure 3C). In both cell lines, GCHD-OE induced significantly higher expression of IDO2 both at mRNA and protein levels (Figures 3D-E).

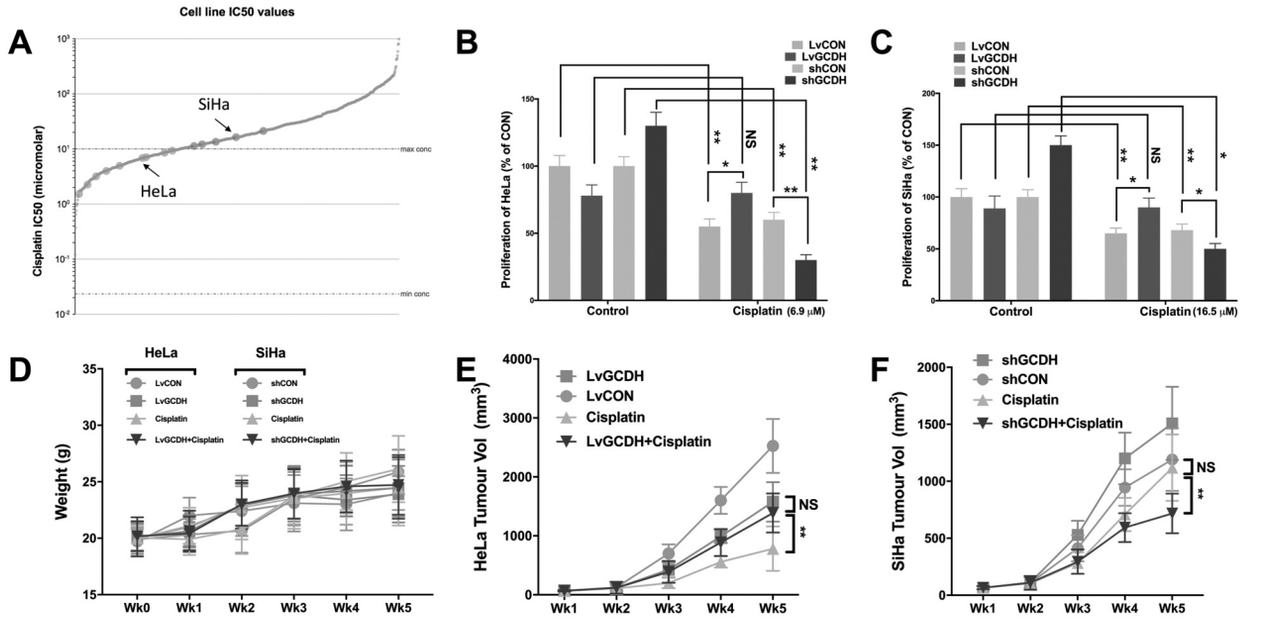

Figure 4. — Association between GCDH and chemoresistance in cervical cancer. A) Reproduced from GDSC dataset; shown are the IC50 curve of cisplatin across a variety of cancer cell lines with highlighted dots representing all cervical cancer cell lines, proliferation at 72 hours from cisplatin treatment in B) HeLa, and C) SiHa cervical cancer cell lines with GCDH overexpression or knockdown. Animal models of nude mice showing: D) tolerable toxicity of cisplatin at low dose (0.5 mg/kg i.p for 7 days), tumor growth curves of E) HeLa, and F) SiHa cells with GCDH overexpression or knockdown, respectively (*$p < 0.05$, **$p < 0.01$).

Here, the authors demonstrated an indirect linkage between GCDH and immune modulation, which could be mediated via IDO2.

Platinum-containing agents remain the mainstay of chemo-regime in cervical cancer. Cell-cycle arrest has been associated with chemoresistance. The authors therefore studied whether GCDH could thus mediate chemoresistance. They first studied baseline response to cisplatin of both cell lines and found that while HeLa was sensitive to cisplatin, SiHa was chemoresistant (Figure 4A). The authors thus decided to use different doses of cisplatin for the treatment to cater for the IC50 of respective cell line. They found that overexpression of GCDH restored inhibitory effect of cisplatin in both cell lines (Figures 4B-C). Of note, GCDH-KD significantly sensitized both cell lines to cisplatin (Figures 4B-C). The authors then examined the chemoresistant effect in animal models using a low-dose cisplatin regime and found that mice did not suffer from intolerable toxicity across all groups (Figure 4D). As HeLa was inherently sensitive to cisplatin and SiHa was inherently resistant, the authors tested GCDH-OE in HeLa and GCDH-KD in SiHa in vivo, respectively. Similar results were also obtained in vivo, showing restored inhibitory effect of GCDH overturned by GCDH-OE for HeLa, whereas resistance overcome via GSDH-KD for SiHa (Figures 4E-F). Here teh authors showed in vitro and in vivo that GCDH could induce chemoresistance in cervical cancer.

Discussion

In the current study, the authors showed that GCDH is overexpressed in cervical cancer. However, its overexpression in cancer is associated with a less aggressive phenotype. In order to exploit the therapeutic potential of GCDH, the authors consider it essential to understand the selective advantage of this less aggressive genotype. Given the findings, the authors speculate that GCDH expression exert protumorigenic effect by mediating immune exclusion and chemoresistance, neither of which has been reported previously as a function of GCDH in cancer.

Immune exclusion plays an important role in cancer development. The authors here demonstrate that GCDH expression is in positive correlation with tumor-infiltrating macrophage. Tumor-associated macrophages are one of the major constituents of tumor stroma in many solid tumors and there is compelling preclinical and clinical evidence that macrophages promote cancer initiation and malignant progression [21]. Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses. Furthermore, the authors demonstrate a strong association between GCDH and IDO2 expression. IDO2 is implicated in tryptophan catabolism and immunity. Contribution of IDO2 to immune escape via DCs and Tregs is similar to IDO1. IDO has been implicated in immune modulation through its ability to limit T cell function and engage mechanisms of immune tolerance. Several clinical trials have proven IDO

inhibitors effective in treating solid tumors [22]. The present findings indicate that targeting GCDH may not only enhance tumor immunity, but also be additive to IDO inhibition in cervical cancer.

Chemoresistance remains a major problem in a variety of cancers. There have been a handful of reports establishing association between cell cycle arrest and chemoresistance. Wiltshire *et al.* reported that in response to irofulven, BRCA1 contributes to the control of S and G(2)/M cell cycle arrest and is critical for repairing DNA double-strand breaks and for RAD51-dependent homologous recombination and BRCA1 deficiency results in increased chromosome damage and chemosensitivity after irofulven treatment [23]. Miwa *et al.* reported that time-lapse fluorescence ubiquitination-based cell cycle indicator imaging demonstrated that both cisplatin could induce cell cycle arrest in S/G2/M in almost all the cells, but a subpopulation of the cells could escape the block and undergo mitosis. The subpopulation which went through mitosis subsequently underwent apoptosis, while the cells arrested in S/G2/M survived. They speculated that chemoresistant cells can be readily identified in a heterogeneous population of cancer cells by S/G2/M arrest, which can serve in future studies as a visible target for novel agents that kill cell-cycle-arrested cells [24]. Brown *et al.* reported that squamous cell carcinomas frequently resist chemotherapy via tumor-propagating cancer cells (TPCs). Quiescent TPCs resist DNA damage and exhibit increased tumorigenic potential in response to chemotherapy, whereas proliferative TPCs undergo apoptosis. Quiescence is regulated by TGF-β/SMAD signaling, which directly regulates cell-cycle gene transcription to control a reversible G1 cell-cycle arrest. Genetic or pharmacological TGF-β inhibition increases the susceptibility of TPCs to chemotherapy because it prevents entry into a quiescent state [25].

In conclusion, the authors have here in part revealed protumorigenic role of GCDH in cervical cancer via the following pathways: (1) upregulation of GCDH may be coexpressed with IDO2, which leads to less infiltrating lymphocytes and (2) GCDH may induce chemoresistance via both induction of tumor-infiltrating macrophages and cell cycle arrest at G2/M phase. These properties of GCDH warrants further investigation. The authors attempted to retrieve clinical information on chemotherapy in the TCGA cervical cancer dataset, yet such documentation was heterogeneous. More rigorous sampling in clinically available cases is now underway in this institute. Conclusively, the present findings support a role of GCDH in cervical cancer. Implication includes potential chemosensitizing effect by targeting GCDH in chemoresistant cases.

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