The Gynecologist

ISSN: 2688-9366

Research Article

Glutathione as a Prognostic Biomarker and a Potential Therapeutic Target for Ovarian Cancer

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Abstract

Aim: It is necessary to find a new prognostic biomarker and therapeutic strategy to improve the prognosis of ovarian cancer as the most lethal gynecologic cancer. Glutathione (GSH) is an antioxidant that protects cells against Reactive Oxygen Species (ROS). We aimed to investigate the clinical significance of GSH and effects of an inhibitor of GSH synthesis, erastin, in ovarian cancer.

Methods: The GSH levels in 41 ovarian cancer tissues were measured with GSH Assay Kit. Survival curves were carried out by the Kaplan-Meier method and evaluated using the log-rank test. Multivariable Cox proportional hazard risk regression model was performed to screen the independent factor affected the prognosis of ovarian cancer patients. In vitro investigations of the effect of erastin were performed using ovarian cancer cell lines. Cell viability, GSH levels and whole (cytosolic and lipid) ROS production were assessed.

Results: Patients with high GSH levels of ovarian cancer tissue had an apparently lower Progression Free Survival (PFS) and Overall Survival (OS) compared with those with low GSH levels. The GSH levels were independent factors for predicting the PFS and OS of ovarian cancer patients. The basal ROS level was inversely proportional to GSH levels in ovarian cancer cell lines. The basal GSH levels were important for estimating the sensitivity to erastin. Reduction of intracellular GSH levels lead increase of whole ROS resulting in cell death.

Conclusions: The GSH levels could become prognostic biomarkers and erastin have a great potential to become a new therapeutic drug in ovarian cancer.

Keywords: Glutathione; Reactive oxygen species; Ovarian cancer; Erastin

Abbreviations

GSH: Glutathione; ROS: Reactive Oxygen Species; WHO: World Health Organization; TNM: Tumor-Node-Metastasis; FBS: Fetal Bovine Serum; PBS: Phosphate Buffered Saline; PFS: Progression Free Survival; OS: Overall Survival

Introduction

The number of patients with ovarian cancer is increasing and deaths due to this disease are also increasing in Japan [1]. Platinum compound is one of the most potent chemotherapy drugs widely used for ovarian cancer [2,3]. Although ovarian cancer is generally sensitive to chemotherapy, most patients ultimately recur and develop resistance to chemotherapy [4,5]. Survival rates are estimated based on previous outcomes [6], but it is not easy to predict what will happen in ovarian cancer patients. Therefore, it is necessary to find a new biomarker to predict prognosis and a new strategy to improve the prognosis of patients with ovarian cancer.

Citation: Takenaka M, Furui T, Suzuki N, Koike T, Aoki H, Morishige K. Glutathione as a Prognostic Biomarker and a Potential Therapeutic Target for Ovarian Cancer. Gynecologist. 2021; 3(1): 1020.

Publisher Name: Medtext Publications LLC

Manuscript compiled: Dec 08th, 2021

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Glutathione (GSH), an antioxidant, is used to mitigate the damage of Reactive Oxygen Species (ROS) [7]. They have roles in cell cycle progression and cell death pathway. Erastin is a classical inhibitor that can lead to the depletion of GSH [8].

In this study, we focused on a potential of GSH as a new prognostic biomarker and erastin as a new strategy in anti-tumor therapies for ovarian cancer.

Materials and Methods

Patients

A total of 41 ovarian cancer tissues were obtained from patients who underwent operation between 2009 and 2018 at Gifu university hospital. The ovarian cancer specimens were subjected to histological examination by 2 expert pathologists for confirmation of World Health Organization (WHO) classification of the tumor, and staging according to the Tumor-Node-Metastasis (TNM) system. The ethics committee of the Gifu University Graduate School of Medicine approved the experiments. Written informed consent was obtained from all patients.

GSH analysis in human ovarian cancer tissues

We performed GSH analysis using the specimen prepared by freezing the tissue separated and sampled from the patient during operation. The tissues were lysed in 5% 5-sulfosalicylic acid dihydrate (Wako Pure Chemical Industries). The lysate was centrifuged (1,000*g) and the supernatant was collected. The supernatant was used to determine the amount of GSH in the sample. We used the GSH and GSSG Assay Kit (product No. S0053, Beyotime) and followed the product instructions to determine GSH levels. Briefly, GSH assay buffer, GSH reductase, 5,5'-dithio-bis 2-nitrobenzoic acid solution and supernatant sample were mixed together and incubated at 25°C

for 5 minutes, then NADPH was added into this system to trigger the reaction. The increase in the absorbance of 5-thio-2-nitrobenzoic acid was measured at 412 nm, and the GSH levels were calculated following the product instructions.

Cell culture

Ovarian cancer cell lines, TOV-21G, KOC-7C (clear cell carcinoma), CaOV3 and SKOV3ip1 (serous carcinoma) were used. KOC-7C cells were provided by the Gynecological and Obstetrics Department, Kurume University and others were provided by the Gynecological and Obstetrics Department, Osaka University [9]. These cells were cultured in Dulbecco's modified medium (Wako) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Wako) fewer than 5% CO_2 at 37°C.

Drug treatments

Treatments of ovarian cancer cell lines with 2.5 μM-20 μM erastin, an inhibitor of GSH synthesis, or 0.5% DMSO as control were performed in Dulbecco' s modified medium containing 10% FBS and 1% penicillin/streptomycin.

Cell viability analysis

Cell viability was evaluated using a Premix WST-1 Cell Proliferation Assay System (TaKaRa Bio Incorporated) according to the manufacturer's instructions. Briefly, cells (1×10^4 cells/well) were seeded in a 96-well plate and treated with different drugs at various concentrations for the indicated times. After addition of 10 μl Premix WST-1 solution to each well, cells were incubated at 37°C for another 1 hour and the absorbance was determined at 440 nm using a microplate reader.

Intracellular GSH analysis

Cells were plated in 6-well plates at a density of 3.0×10^5 cells/well and cultured overnight. Cells were received different treatment for 4 hours followed by harvesting to determine cell number. Nearly 6×10^4 live cells from each sample were transferred to new tubes, washed in Phosphate Buffered Saline (PBS) and centrifuged at 1200 rpm at 4°C for 5 minutes twice. The cell pellet was resuspended in 80 μl protein removal solution, thoroughly incorporated, and placed in -70°C and 37°C sequentially for fast freezing and thawing, then placed in 4°C for 5 minutes and centrifuged at 10,000 *g for 10 minutes. The supernatant was used to determine the amount of GSH in the sample. We used the GSH and GSSG Assay Kit and followed the product instructions to determine GSH levels.

Analysis of ROS production

Cells were plated in 10 cm dishes at a density of 1.0×10^5 cells/well and cultured overnight. After treated with test compounds for 15 hours, harvested in 5 ml Dulbecco's modified medium containing Deep Red Reagent (5 μM) (Molecular Probes, Invitrogen) and incubated for 30 min at 37°C in a tissue culture incubator. After trypsinized, cells were resuspended in 3% FBS in PBS and strained through a 40 mM cell strainer (BD Falcon). Cells were analyzed using a flow cytometer (FACSAria, BD Biosciences) equipped with 488 nm laser for excitation. Data were collected from the Deep Red Reagent channel (MitoSOX). A minimum of 1.0×10^4 cells were analyzed per condition.

Statistical analysis

Data are expressed as means ± SD of 3 independent experiments and were evaluated using an ANOVA LSD test. Student *t* test was conducted for intergroup comparison. Pearson correlation test was used for correlation analysis. Survival curves were generated with Kaplan-Meier plots. The results are presented with P values from a log-rank test. Multivariable Cox proportional hazard risk regression model was performed to screen the independent factor affected the prognosis of ovarian cancer patients. All tests were 2-sided, and P values <0.05 were considered statistically significant.

Results

Patient characteristics

The GSH levels in 41 ovarian cancer tissues were measured. The GSH levels were considered as either low (n=34) or high (n=7) according to the cut-off value, which was defined as the median of the cohort. The GSH levels were 73.1 ± 74.1 and 669.1 ± 339.5 μmol/l in each low and high group (Table 1). There was no significant difference in all parameters (e.g., age, type of pathology, stage, type of surgery, neoadjuvant and adjuvant chemotherapy).

Table 1: Characteristics of ovarian cancer patients who received GSH levels assay.

		GSH Levels		
		low	high	*P*-value
Cases (n)		34	7	
GSH levels (average ± SD)		73.1 ± 74.1	669.1 ± 339.5	<0.01
age (average ± SD)		60.0 ± 11.0	60.0 ± 16.5	0.55
type of pathology	HGSC	13	5	0.26
	LGSC	1	0	
	CCC	17	1	
	EC	1	1	
	MC	2	0	
Stage	I+II	17	3	0.73
	III+IV	17	4	
Type of surgery	complete and optimal	30	6	0.85
	others	4	1	
NAC	+	13	3	0.82
	-	21	4	
adjuvant chemotherapy	+	31	7	0.41
	-	3	0	

HGSC: High Grade Serous Carcinoma; LGSC: Low Grade Serous Carcinoma; CCC: Clear Cell Carcinoma; EC: Endometrioid Carcinoma; MC: Mucinous Carcinoma; NAC: Neoadjuvant Chemotherapy

High GSH levels predict poor prognosis in ovarian cancer

To investigate the clinical significance of GSH, we assessed the association GSH levels and prognosis in ovarian cancer. The Kaplan-Meier analysis was performed to determine the prognostic value of GSH in ovarian cancer patients. We performed a log-rank test which compared patients with higher GSH levels with those with lower levels. Patients with higher GSH levels had significantly shorter Progression Free Survival (PFS) than those with a lower GSH levels (P<0.01) (Figure 1). It also showed that the Overall Survival (OS) was shorter in ovarian cancer patients with higher GSH levels (P<0.001) (Figure 2).

GSH levels as independent factors for predicting the prognosis of ovarian cancer

We performed the multivariate Cox regression analysis to show that GSH levels were independent factors predicting the prognosis. GSH levels were independent factors for both PFS (HR=5.330, 95% CI: 1.334-21.291; P=0.018) and OS (HR=7.174, 95% CI: 1.572-32.738; P=0.011) (Tables 2 and 3).

The sensitivity to an inhibitor of GSH synthesis, erastin, in ovarian cancer cell lines

Next, we demonstrated *in vitro* investigation using ovarian cancer

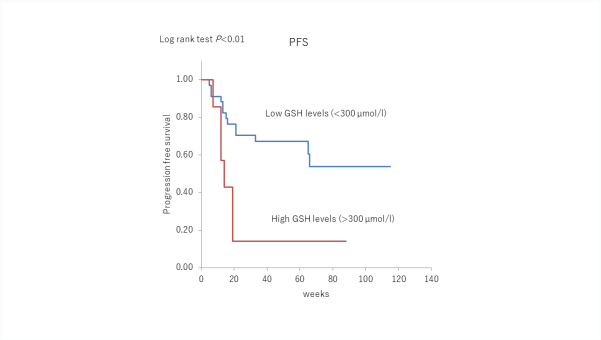

Figure 1: The Kaplan-Meier progression free survival determined by GSH levels of ovarian cancer patients. According to the GSH levels, Kaplan-Meier progression free survival of ovarian cancer patients showed that high GSH levels were significantly correlated with poor prognosis (P<0.01).

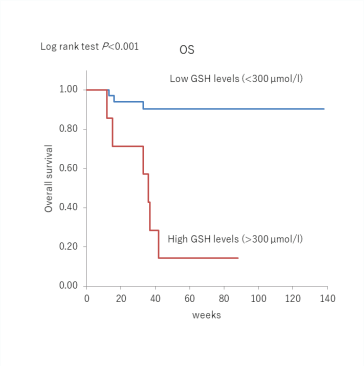

Figure 2: The Kaplan-Meier overall survival determined by GSH levels of ovarian cancer patients. According to the GSH levels, Kaplan-Meier overall survival of ovarian cancer patients showed that high GSH levels were significantly correlated with poor prognosis (P<0.001).

Table 2: Multivariate analysis for progression free survival by Cox regression model.

Variable	Hazard ratio	95% CI			*P*-value
GSH levels	5.33	1.334	-	21.291	0.018
age	0.992	0.953	-	1.032	0.683
type of pathology	3.244	0.839	-	12.539	0.088
stage	2.526	0.374	-	17.061	0.342
type of surgery	0.233	0.045	-	1.198	0.081
NAC	2.833	0.675	-	11.882	0.155
adjuvant chemotherapy	1.632	0.066	-	40.081	0.764

CI: Confidence Interval; NAC: Neoadjuvant Chemotherapy

Table 3: Multivariate analysis for overall survival by Cox regression model.

Variable	Hazard ratio	95% CI			*P*-value
GSH levels	7.174	1.572	-	32.738	0.011
age	0.976	0.926	-	1.028	0.354
type of pathology	3.834	0.972	-	15.126	0.055
stage	4.44	0.541	-	36.439	0.165
type of surgery	0.17	0.03	-	0.974	0.047
NAC	3.191	0.66	-	15.437	0.149
adjuvant chemotherapy	1.049	0.028	-	39.331	0.979

CI: Confidence Interval; NAC: Neoadjuvant Chemotherapy

cell lines focused on GSH. Erastin is identified as an inhibitor of GSH synthesis. To determine whether erastin induces growth inhibition in ovarian cancer cells, TOV-21G, KOC-7C, CaOV3 and SKOV3ip1 cells were treated with erastin for 24 hours and cell viability was assayed using Premix WST-1 assay (Figure 3). Comparatively, TOV-21G and SKOV3ip1 cells were more sensitive to erastin than CaOV3 and KOC-7C cells.

The accumulation of ROS induced by GSH depletion

We compared erastin sensitive ovarian cancer cell line, TOV-21G, with less sensitive cell line, KOC-7C, focused on the basal GSH and ROS level. Although the GSH levels were reduced by erastin both in TOV-21G and KOC-7C cell, the basal GSH levels in KOC-7C cells were higher than that in TOV-21G cells under control condition (Figure 4). We observed that treatment with erastin resulted in increase of ROS in both cell lines but the basal ROS level in KOC-7C cell was lower than that in TOV-21G cell (Figure 5). Erastin decreased intracellular GSH levels and increased ROS resulting in cell death in ovarian cancer cells. The sensitivity to erastin was depends on intracellular basal GSH levels.

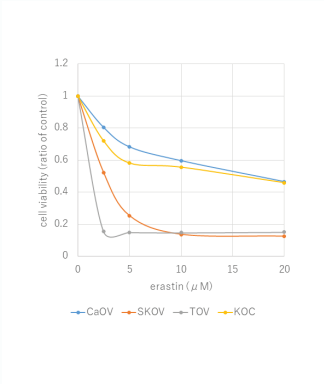

Figure 3: The sensitivity to erastin in ovarian cancer cell lines. Ovarian cancer cell lines (CaOV, SKOV, TOV-21G and KOC-7C) were treated with erastin for 24 hours and cell viability was assayed using Premix WST-1 assay.

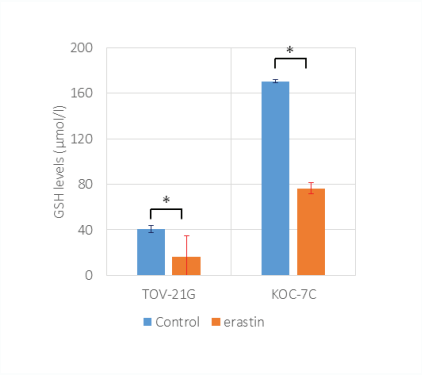

Figure 4: GSH levels influenced by erastin. TOV-21G and KOC-7C cells were treated with erastin (10 μM) for 4 hours and GSH levels were assayed using GSSG/GSH Quantification Kit. *P< 0.05, Student's t test.

Discussions

Ovarian cancer remains the most lethal gynecologic cancer [10]. Although several studies evaluated serum tumor markers (e.g., CA-125, CA19-9 and CEA) in ovarian cancer, prognostic biomarkers are little known [11]. Prognostic biomarkers are useful to identify patients at different levels of risk. We focused on a prognostic biomarker and a new therapeutic strategy for ovarian cancer.

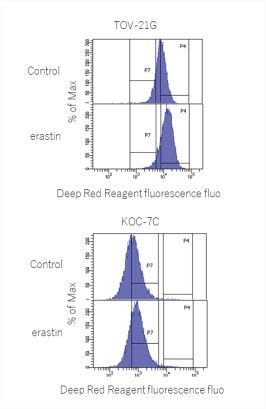

Figure 5: ROS level influenced by erastin. TOV-21G and KOC-7C cells were treated with erastin (10 μM) for 15 hours. Following, whole (cytosolic and lipid) ROS production were assessed by flow cytometry using Deep Red Reagent.

Oxidative stress has long been implicated in cancer development and progression [12], suggesting that antioxidant treatment may provide protection from cancer [13]. Among the enzymatic systems involved in the maintenance of the intracellular redox balance, a main role is played by GSH that participates not only in antioxidant defense systems but also in many metabolic processes [14,15]. GSH is a tripeptide formed by glutamic acid, cysteine, and glycine. In many normal and malignant cells, increased GSH levels are associated with a proliferative response and essential for cell cycle progression [16,17]. On the other hand, GSH depletion regulates the activation of cell death pathways [18].

Thus, several studies about biochemical function of GSH were published. However, their potential utility in clinical practice as prognostic biomarkers is unknown. To investigate the clinical features of the GSH status, we assessed the associations between GSH levels and prognosis of ovarian cancer patients. The GSH levels in ovarian cancer tissues of 41 patients were measured. Interestingly, ovarian cancer patients with high GSH levels had an evidently lower PFS and OS compared with those with low GSH levels. Notably, GSH levels were independent factors for predicting the PFS and OS of ovarian cancer patients. We investigated that elevated GSH levels lead antioxidant defense systems resulting in defense to evade cancer cell death and develop drug resistance. Our data indicated that GSH might be a potent biomarker for predicting the prognosis of ovarian cancer patients.

Next, we demonstrated *in vitro* investigation to assess the oxidant system associated with GSH levels in ovarian cancer. Recently, Qi Cheng et al. [19] reported that erastin works synergistically with cisplatin to inhibit ovarian cancer cell growth. Erastin induced the depletion of GSH resulting in increase of lipid ROS [20,21]. In detail, erastin inhibits system Xc^- resulting in cysteine starvation and ferroptotic cell death [22,23].

Our data showed that the basal GSH levels were lower in erastin sensitive ovarian cancer cell lines than less sensitive cell lines and the basal ROS level was inversely proportional to GSH levels in both cell lines. Although the depletion of GSH induced by erastin leads to ROS in both cell lines, cell death could not be lead in less sensitive ovarian cancer cell lines because the basal ROS level was too low. We showed that the basal GSH levels are important for estimating the sensitivity to erastin. GSH, an antioxidant, could have a potential to be targeted for ovarian cancer treatment.

Conclusion

Our study demonstrated that high GSH levels were significantly associated with a poor prognosis independently of other factors in ovarian cancer. Erastin leads ovarian cancer cell death depends on intracellular GSH levels. This is the first report to show a potential of GSH as a prognostic biomarker and a new target of ovarian cancer treatment.

Acknowledgement

We gratefully acknowledge the work of past and present members of our laboratory.

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