THE REGULATION OF PLASMA GELSOLIN BY DNA METHYLATION IN OVARIAN CANCER CHEMORESISTANCE

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ABSTRACT

Ovarian cancer (OVCA) is the most lethal gynecologic cancer. Chemoresistance remains a major hurdle to successful therapy and patient survival. The secreted isoform of the actin-associated protein, gelsolin (plasma gelsolin; pGSN), is highly expressed in chemoresistant than chemosensitive OVCA cells, although the mechanism underlying the differential expression is not known. Also, its overexpression significantly correlates with shortened survival of OVCA patients. DNA methylation plays a key role in the regulation of genes expression and contributing to cancer development and chemoresistance with the help of DNA methyltransferases (DNMTs) or Ten eleven translocation (TETs) enzymes. TET1 is the most studied isoform of TETs family and primarily responsible for 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) oxidation to initiate demethylation and increase in the expression of methylated genes. Whether pGSN expression in OVCA cells is regulated by DNA methylation and TET1 regulates the differential pGSN expression between chemosensitive and resistant OVCA cells is not known. In this study, we hypothesized pGSN overexpression in chemoresistant OVCA cells is due to the hypomethylation at its promoter region by TET1. Our objective was to investigate whether DNA methylation and specifically TET1 plays a role in the regulation of differential pGSN expression and chemosensitivity in OVCA. Chemosensitive and resistant OVCA cell lines of different histological subtypes were used in this study to measure pGSN and TET1 mRNA abundance and protein contents by qPCR and Western blotting respectively. Cisplatin-induced chemoresponsiveness was morphologically assessed by Hoechst staining (apoptosis). Infinium HumanMethylation450 BeadChip assay was used for global methylation analysis of twelve (12) different OVCA cells and to investigate the role of DNA methylation specifically in pGSN regulation and pGSN-induced chemoresistance. DNMTs and

TETs were pharmacologically inhibited in sensitive and resistant OVCA cell using specific inhibitors. Gain-and-loss-of-function assays were carried to identify the relationship between TET1 and pGSN in OVCA chemoresponsiveness. Differential protein and mRNA expressions of pGSN and TET1 were observed between sensitive and resistant OVCA cells and cisplatin reduced their expression in sensitive but not in resistant cells. Global methylation analysis revealed hypomethylation in resistant cells compared to sensitive cells. Pharmacological inhibition of DNMTs increased pGSN protein levels in sensitive OVCA cells and decreases their responsiveness to cisplatin, however we did not observe any difference in methylation level at pGSN promoter region. TETs inhibition resulted in hypermethylation at multiple CpG sites and decreased pGSN protein level in resistant OVCA cells which was also associated with enhanced response to cisplatin, findings that suggested the methylation role of TETs in the regulation of pGSN expression in OVCA cells. Further, we found that TET1 is inversely related to pGSN and positively related to chemoresponsiveness of OVCA cells. This project does not only broaden our knowledge about the mechanistic insights into the epigenetic regulation of pGSN in OVCA chemoresistance, but it also reveals a new potential target to re-sensitize chemotherapy resistant OVCA cells. This may provide a future strategy to improve overall OVCA patient survival.

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ABBREVIATIONS

5caC – 5-Carboxycytosine

5-fc – 5-formylcytosine

5-FU – 5-Fluorouracil

5-hmC – 5-Hydroxymethylcytosine

5mC – 5-Methylcytosine

CCA – Cholangiocarcinoma

CCC – Clear Cell Carcinoma

CCK-8 – Cell Counting Kit-8

cGSN – Cytoplasmic Gelsolin

CRABP2 – Cellular Retinoic Acid Binding Protein 2

DMRs – Differentially Methylated Regions

DMSO – Dimethyl Sulfoxide

DNMT – DNA Methyltransferases

ECL – Enhanced Chemiluminescent

EMT – Epithelial to Mesenchymal Transition

EOC – Endometrioid Ovarian Carcinoma

EOC – Epithelial Ovarian Cancer

ESCs- Embryonic Stem Cells

FAK – Focal Adhesion Kinasae

FIGO – International Federation of Gynecology and Obstetrics

FLICE – Fas-Associated Death Domain-Like Interleukin-1beta-Converting Enzyme

FLIP – FLICE-like Inhibitory Protein

GSN – Gelsolin

HBOC – Hereditary Breast and Ovarian Cancer

HDACi – Histone Deacetylases Inhibitor

HDACs – Histone Deacetylases

HGSOC – High-Grade Serous Ovarian Carcinoma

HIF-1α – Hypoxia-Inducible Factor-1

HIPEC – Hyperthermic Intraperitoneal Chemotherapy

HO-1 – Heme Oxygenase-1

HRP – Horseradish Peroxide

IDS – Interval Debulking Surgery

IDT – Integrated DNA Technology

IFNγ – Interferon Gamma

LGSOC – Low- Grade Serous Ovarian Carcinoma

MeCP – Methyl Cytosine Binding Proteins

MET – Mesenchymal to Epithelial Transition

MGMT – o6-methylguanine-DNA methyltransferase

miRNA – microRNA

MOC – Mucinous Ovarian Carcinoma

Nrf2 – Nuclear Factor-Erythroid 2-Related Factor 2

OS – Overall Survival

OSCC – Oral Squamous Cell Carcinoma

OVCA – Ovarian Cancer

PARP – Poly (ADP)-Ribose Polymerase

PBS – Phosphate-Buffered Saline

PD-1 – Program Death Ligand

PDS – Primary Debulking Surgery

PFI – Progression Free Interval

PFS – Progression Free Survival

pGSN – Plasma Gelsolin

PIP_2 – Phosphatidylinositol-4-5-bisphosphate

PPS – Partially Platinum Sensitive

qPCR – Quantitative Polymerase Chain Reaction

RASSF5 – Ras Association Domain Family Member 5

SAP – Shrimp Alkaline Phosphatase

TAMs – Tumor-Associated Macrophages

TDG – Thymine DNA Glycosylase

TET – Ten-Eleven Translocation Enzymes

TNFα – Tumor-Necrosis Factor Alpha

UTR – Untranslated Region

VEGFA – Vascular Endothelial Growth Factor A

α-KG – Alpha-Ketoglutarate

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1. INTRODUCTION

1.1 Ovarian Cancer

i. Epidemiology, Etiology and Risk Factors:

Ovarian cancer (OVCA) is the third most common gynaecological cancer after cervical and uterine cancer. Although OVCA is not the most prevalent cancer among females, it ranks first in gynaecological cancer related deaths. In 2020, there were a total 313,959 new OVCA cases reported globally accounting 3.4% of all cancers with 207,252 recorded deaths accounting 4.7% of cancer related deaths[1]. OVCA is broadly classified into epithelial, sex-cord stromal, germ cell and mixed-cell type based on the tumor origin. Epithelial ovarian cancer (EOC) is the most common and one of the well-characterised types of ovarian cancer, accounting for approximately 90% of all OVCA cases and originates from the surface epithelium of the ovary[2]. EOC is further sub-classified into five histotypes including high-grade serous ovarian carcinoma (HGSOC), low-grade serous ovarian carcinoma (LGSOC), endometrioid ovarian carcinoma (EOC), clear cell carcinoma (CCC) and mucinous ovarian carcinoma (MOC) and are different from each other based on the tumor origin and cell morphology. These histotypes have further subtypes based on their molecular profile, risk factors and clinical presentation[3,4] **(Fig. 1)**.

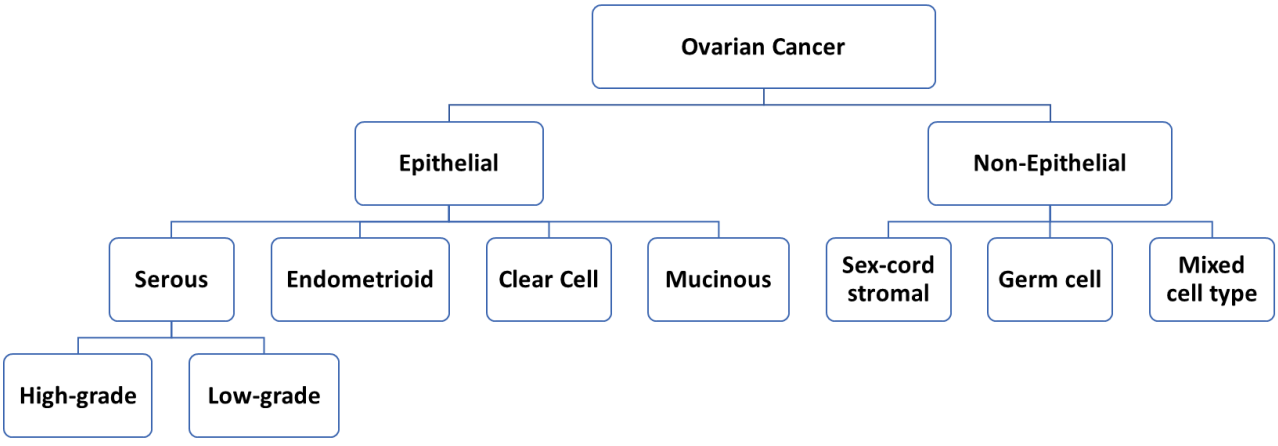

Figure 1. Classification of ovarian cancer. OVCA is broadly classified into four types which are further sub-classified based on the tumor origin and cell morphology.

OVCA is often called a “silent cancer” due to its asymptomatic nature and late presentation. Around 60% of the OVCA cases are diagnosed at the advanced stage when disease has significantly progressed, explaining the highest mortality rate of this disease[5]. OVCA patients present with unspecific symptoms (abdominal bloating, stomach problems including diarrhea or constipation, urinary symptoms, dyspareunia, early satiety, nausea, pelvic pain, fatigue, and weight loss) which could lead to misdiagnosis[6]. Despite the advances in diagnosis and treatment approaches, the 5-year survival rate remains between 30-40% for stage III and as low as 20% for stage IV OVCA patients. The overall survival generally ranges between 30-40% around the globe and has experienced only a slight increase since the last two decades[7].

OVCA is a heterogeneous group of histotypes, with many associate risk factors. Some of these risk factors may involve hereditary, demographics, hormonal changes, lifestyle habits, reproductive age, and environmental factors **(Table 1)**. OVCA is predominantly found in postmenopausal women where age is directly related to disease aggressiveness, higher incidences, and lower survival rates, however positive family history of breast and ovarian cancer is the most significant risk factor for ovarian cancer[2,8].

Table 1. Risk factors for OVCA

Factors	Risk Factors	Protective Factors	Controversial
Hereditary	Family history Hereditary Breast and Ovarian Cancer (HBOC) syndrome Lynch syndrome		
Demographic	Age		
Hormonal	Estrogen Androgen	Contraceptive methods Progestin	Hormonal replacement therapy Infertility treatment
Gynaecological	Endometriosis Polycystic ovarian syndrome	Hysterectomy Tubal ligation	Pelvic inflammatory disease
Reproductive	Menstrual related factors Late menopause	Multiparity Breastfeeding Age at childbirth	Young menarche
Lifestyle, environmental, diet and other	Radiation exposure Psychotropic medication Perineal talc exposure Lower socioeconomic status		Nutrition and diet Alcohol, caffeine, and cigarettes High level physical activities, high body-mass-index

Cited and modified from[8–10]

ii. Treatment strategies

The first line of treatment for OVCA is the combination of surgical debulking and chemotherapy since the 1980s. Newly diagnosed ovarian cancer cases are conventionally treated primarily by surgical tumor staging, followed by debulking surgery and platinum based chemotherapy treatment[11]. Over the last decade, significant advances have been made in the treatment of OVCA, with immunotherapies and targeted therapies gaining considerable attention,

although minimal therapeutic success. Recently, Poly(ADP)-ribose polymerase (PARP) inhibitor (PARPi) therapy has been included in first line treatment for HGSOC and platinum-sensitive relapsed patients[12,13].

a) Surgery

Once OVCA is diagnosed, surgery allows physicians to accurately stage the tumor according to the International Federation of Gynecology and Obstetrics (FIGO)[14].

In case of early stage OVCA, surgical staging includes peritoneal cavity inspection, bilateral salpingo-oophorectomy, hysterectomy, omentectomy, retroperitoneal lymph node dissection in pelvic and para-aortic regions and peritoneal biopsy[15]. In advanced stage OVCA, a extended cytoreduction (primary debulking surgery; PDS) including bowel resection, splenectomy, diaphragm resection, and pelvic/abdominal peritonectomy is performed to eliminate all macroscopic cancer lesion with thickness >1cm (residual disease)[16]. Optimal debulking surgery with no residual disease is a powerful independent determinant of improved overall survival[17]. In advanced stages, where complete cytoreduction could not be performed due to disease metastasis or patient's condition, interval debulking surgery (IDS) is performed after a few cycles of neoadjuvant chemotherapy administration to reduce the tumor burden[18].

b) Chemotherapy

OVCA patients receive platinum-based chemotherapy after surgery based on tumor stage, presence of residual disease, histology and the molecular profile of the cancer cells[19]. Platinum (pt)-based drugs act by covalently binding to DNA leading to the formation of pt-(GpG) di-adducts, which inhibit DNA replication, impede cell proliferation, and activate DNA damage response. DNA damage response initiated by platinum drugs results in the activation of p53, p73 and mitogen-activate protein kinases resulting in apoptosis and ultimately cell death[20].

Over the last two decades, the primary chemotherapy regimen for OVCA patients is the combination of carboplatin and paclitaxel every three weeks for six cycles[21]. The preferred administration route of these drugs is intravenous, however in some clinical studies, the intraperitoneal administration route provided a much significant overall survival due to tumor localization in the peritoneal cavity[22,23]. Hyperthermic intraperitoneal chemotherapy (HIPEC) is

another promising approach for OVCA treatment which involves peritoneal profusion of heated chemotherapy at the time of debulking surgery[24]. Hyperthermia has been found effective in enhancing chemotherapy drugs penetration and chemosensitivity by impeding DNA repair mechanisms, inducing apoptosis, modifying immune response via natural killer cells activation, and inhibiting angiogenesis[25,26]. In a randomised clinical trial, patients who have undergone IDS and given chemotherapy with HIPEC (verses without HIPEC) experienced increased disease-free and overall survival by 12 months. Additionally, only minimized adverse side effects were recorded[24].

c) Molecular Targeted Therapies

In recent years, anti-cancer targeted therapies have greatly improved the effectiveness of first-line treatment in different cancer types including ovarian cancer. So far, two targeted therapies, anti-angiogenic and PARPi, have been approved by FDA as first-line treatments of advanced ovarian cancer cases or in platinum sensitive relapses[12,27–31].

Bevacizumab, a humanized monoclonal antibody, is an anti-angiogenic therapeutic agent that targets and neutralises vascular endothelial growth factor A (VEGFA) as well as inhibits tumor angiogenesis that drives metastasis[32]. Two independent clinical trials; GOG218 and ICON7 have reported significant increase in progression-free survival when bevacizumab was administered in combination with platinum based chemotherapy as concurrent and maintenance therapy[33,34]. In 2018, FDA eventually approved bevacizumab as a first-line treatment for advanced stage ovarian cancer. Interestingly, updated results from GOG218 revealed no improvement in overall survival with bevacizumab as a standard regimen of ovarian cancer.[35]. Phase III results of the ICON7 trial showed overall survival increase in advanced ovarian cancer patients[36]. Adverse side effects were also observed with the use of bevacizumab including haemorrhage, proteinuria, thrombosis, bowel perforation and hypertension[37].

Recently, PARPi has emerged as another promising treatment option for ovarian cancer patients. PARP enzymes play an important role in DNA damage repair, and if inhibited results in stalled replication, and accumulation of double strand breaks which is repaired by homologous recombination repair machinery[38]. In 2019, based on SOLO-1 phase III study, the first PARPi (Olaparib) was approved by FDA to be used as a front-line maintenance therapy for advanced

stage ovarian cancer patients with BRCA1/BRCA2 mutations. A 70% decrease in disease progression and death was reported in the patient cohort that received Olaparib compared to placebo group[13]. Further clinical trials in advanced OVCA patients (initially responsive to standard chemotherapy) combining Olaparib with bevacizumab (PAOLA-1 phase III trial), Veliparib (VELIA trial), and Niraparib maintenance therapy (PRIMA trial) reported significant improvement in progression free survival[12,31,39].

d) Immunotherapy

Although surgery and chemotherapy have always been the conventional approach for ovarian cancer treatment, the efficacy of immunotherapy has been tested in many clinical trials. Administration of immune checkpoint inhibitors targeting program death ligand (PD-1; nivolumab or pembrolizumab/PD-L2; atezolizumab) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4; ipilimumab and tremelimumab) have shown modest response including increased toxicity and progression-free survival, and decreased CA-125 level in 15% of OVCA patients[40–43]. Currently, there are ongoing trials using single immunotherapy agents (NCT02608684, NCT02440425, NCT02537444, Keynote-100/NCT02674061, NCT01611558) or their combination with other therapies including platinum chemotherapy, PARPi, and anti-angiogenic therapy (NCT02865811, NCT02853318, Javelin ovarian 100/NCT02718417, Javelin ovarian 200/NCT02580058, EORTC-1508/NCT02659384, NCT02484404) as treatment for ovarian cancer[44].

iii. Chemotherapy Resistance

Most of ovarian cancer patients show significant clinical response to first-line chemotherapy, however, 25% of early stage and 80% of advanced stage ovarian cancer patients relapse with chemoresistance, causing over 90% of deaths[45,46]. Due to high recurrence rate and chemoresistance development, the 5-year survival rate of advanced stage OVCA patients is as low as 20%[2]. Progression free interval (PFI), the time period from last platinum-based therapy to recurrence, is a reliable indicator for stratifying recurrent ovarian cancer patients to chemosensitive (PFI>12 months), partially platinum-sensitive (PPS) (PFI within 6-12 months) or chemoresistant (PFI<6) groups[47,48]. Although the exact mechanisms of platinum resistance are multifactorial and may involve one or more of the following etiologic factor: alteration of

multiple molecular pathways including mutation and silencing of tumor suppressor genes, activation of oncogenes, epigenetic modifications (DNA methylation, histone modifications, chromatin remodeling, miRNA modifications), dysregulation of pro-apoptotic (PI3K/Akt) and anti-apoptotic signalling pathways (Bcl-2, Bcl-xl, p53), tumor heterogeneity, epithelial to mesenchymal transition (EMT) (increased cell migration and invasion; metastasis), dysregulation in drug uptake and efflux, involvement of tumor microenvironment, increased DNA damage repair and the upregulation of gelsolin expression and function[49–56]. Therefore, chemoresistance development has been a major challenge in ovarian cancer treatment.

1.2. Epigenetic Modifications and OVCA Chemoresistance

Epigenetic modifications are molecular mechanisms that modify gene expression without introducing any changes to DNA sequence[57]. Under normal circumstances, these modifications regulate and ensure normal genome functioning, whereas epigenetic aberrations often result in disease development such as cancer[57,58]. Histone modification, microRNA (miRNA) modifications and DNA methylation are the most studied epigenetic mechanisms that are involved in ovarian cancer development, progression and chemoresistance.

i. Histone modifications

Histone modifications regulate gene expression by altering the chromatin structure resulting in cancer development[59]. Chromatin is a compact structure of nucleosome repeats that are bridged by linker DNA. Each nucleosome consists of DNA strands (~146 bp long) coiled around histone protein octamers (pair of four histone proteins: H2A, H2B, H3, H4)[60]. Gene regulation by histone is achieved through post-translational modifications (acetylation, phosphorylation, ubiquitination, methylation, SUMOylation, adenosine diphosphate ribosylation) of histone tail (NH_2 terminal of histone protein)[61–63]. This is reversibly controlled by a group of enzymes including histone acetyltransferases/deacetylases, phosphatases, ubiquitin ligases/deubiquitinates, methyl/de-methyltransferases, SUMO ligases, and proteases[61,64]. Dysregulation of these enzymes' activities shift the gene expression balance and causes gain/loss of function, activation of oncogenes, dysregulation of tumor suppressor genes, and chromosomal translocation that ultimately leads to the pathogenesis of human cancers[65–67].

Increased enzymatic activity of histone deacetylases (HDACs) is often associated with chemoresistance development in ovarian cancer patients. The increased expression of the first three members of the HDAC family (1,2,3) is significantly associated with higher mitotic index rate in HGSOC patients[68]. Additionally, the overexpression of these isoforms in OVCA cells was also associated with cisplatin chemoresistance development[69]. HDAC1 and HDAC7 are highly expressed in ovarian cancer stem cells[70] and their overexpression is associated with tumor relapse and metastasis. Meanwhile, treatment with FDA approved HDAC inhibitor (vorinostat) decreased the expression of HDAC7[71]. Improved sensitivity to cisplatin or heat shock protein (HSP90) treatment has been reported in ovarian cancer cells when either of the treatments was combined with HDAC inhibitor (HDACi)[72]. Knocking down of HDAC1 resulted in decreased expression of c-Myc (protooncogene) and upregulation of miR-34a (tumor suppressor miRNA) which led to increased chemo-responsiveness of resistant ovarian cancer cells[73]. These findings suggest that histone modifications play an important role in ovarian cancer chemoresistance and could potentially be targeted to develop improved therapeutic approaches for ovarian cancer.

ii. microRNAs modifications

miRNAs are small noncoding single stranded RNAs (~22 nucleotides in length) which are associated with post-transcriptional regulation of ~60% of genes associated with various cellular and biological processes[74]. miRNAs negatively regulate the protein expression by binding to 3`-untranslated region (UTR) of target messenger RNA to inhibit its translation or impairing the stability resulting in its degradation[75]. A single miRNA is able to regulate hundreds of mRNA as it does not necessarily require complete base-pair complementarity. Similarly, one mRNA can be regulated by a number of different miRNA[76]. miRNAs can either be oncogenic or tumor suppressor in nature depending on the target gene and contribute to cancer development, progression, and chemoresistance[77,78].

The miR-200 family (miR-200a, miR-200b, miR-200c), miR-141 and miR-429 have recently been the focus of attention in ovarian cancer chemoresistance development. Increased expression of miR-200a in ovarian cancer tissues is significantly associated with improved response to paclitaxel-based chemotherapy, whereas its low expression is associated with poor progression-free survival[79]. A previous study has demonstrated that the inhibition of miR-200a and miR-141 triggered EMT and platinum-induced resistance in ovarian cancer sensitive cells. However, the

overexpression of these two miRNAs in resistant ovarian cancer cells resulted in the induction of mesenchymal to epithelial transition (MET) and increased sensitivity to paclitaxel and carboplatin therapy[80]. Similar results were observed in studies where miR-574-3p promoted sensitivity to paclitaxel and cisplatin treatment. Overexpression of miR-574-3p inhibited metastasis by suppressing EGFR and inhibiting its downstream targets including AKT, FAK and c-Src which controls signaling pathways associated with cell survival, cell cycle, cellular proliferation, and migration[81].

miR-136 is downregulated in ovarian carcinoma patients compared to control patients and its downregulation is associated with poor prognosis of patients[82]. The overexpression of miR-136 in chemoresistant OVCA cells downregulates Notch3 signaling, resulting in decreased cell survival and increased chemosensitivity[82]. The role of circulating miRNAs have also been studied in different cancers and they were identified as potential biomarker for cancer pathogenesis, progression and chemoresistance[83–87].

iii. DNA methylation

DNA methylation is a well-known epigenetic mechanism associated with gene regulation in cancer development, progression, recurrence and chemoresistance. DNA methylation mechanism is regulated by two sets of enzymes, namely, DNA methyltransferase family enzymes (DNMT1, DNMT3A, DNMT3B) and Ten-eleven translocation methylcytosine dioxygenase family (TET1, TET2, TET3). DNMT introduces methylation by converting carbon 5 of cytosine residue which is usually followed by guanine in CpG dinucleotides to 5-methylcytosine (5mC) by the addition of methyl group[88]. In contrast, TET actively catalyzes the hydroxy-methylation by converting 5-methylcytosine to 5-hydro-methylcytosine (5-hmC) and facilitate de-methylation of DNA **(Fig. 2)** at CpG islands and cysteine-rich sites with further help of thymine DNA glycosylase (TDG) proteins[89–91]. Methylated cytosine in the promoter region binds to methyl cytosine binding protein (MeCP) which inhibits the promoter recognition by transcription factor and RNA polymerases and thus suppressing the transcription of targeted gene[92]. On the other hand, decreased methylation of CpG sites at the promoter region results in increased gene expression[58]. Downregulation of tumor suppressor genes by aberrant methylation in their promoter region and global hypomethylation/specific hypomethylation of oncogenes are two most common epigenetic phenomena occurring in all cancers, including ovarian cancer[58,93].

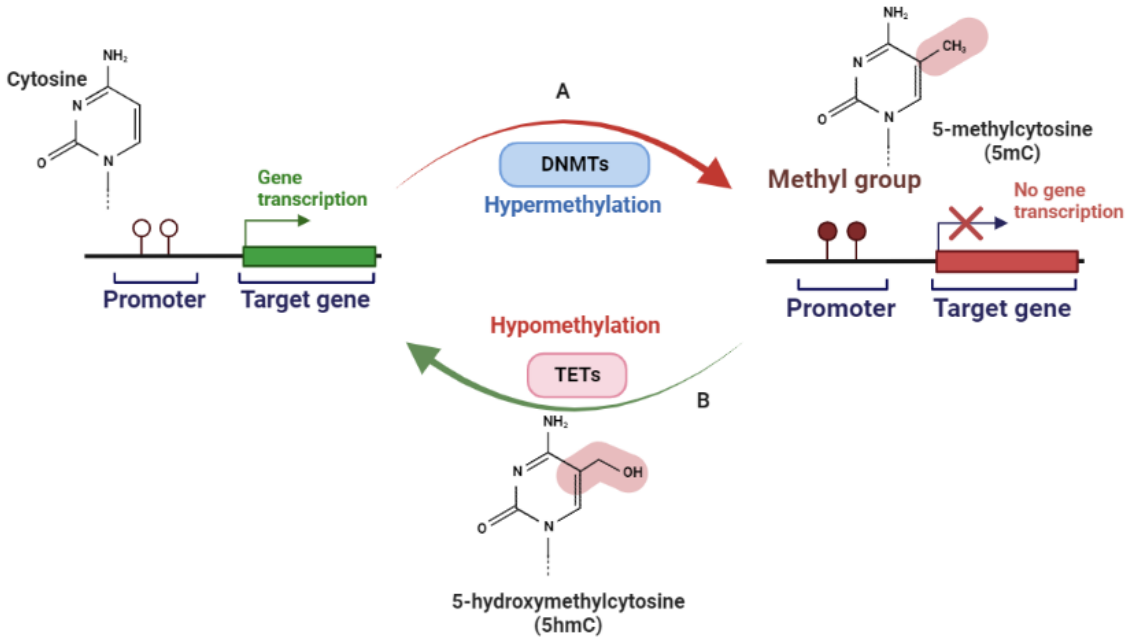

Figure 2. **Gene regulation by DNA methylation mechanism.** Gene expression is regulated by DNA methylation, which is controlled by DNMTs and TETs enzymes. **(A)** DNMTs introduces hypermethylation, by converting carbon 5 of cytosine residue to 5mC, within gene promoter region and inhibit the promoter recognition and in turn transcription of targeted gene. **(B)** TETs actively catalyzes demethylation by introducing hydroxy-methylation of 5mC and converting it to 5-hmC which is further oxidized to demethylated cytosine and thus reactivate the targeted gene transcription. (Figure designed using BioRender)

The role of altered DNA methylation, both at specific gene and the global level, in the ovarian cancer chemoresistance development has extensively been explored. Whole epigenome profiling of 80 primary ovarian cancer tissue samples were performed to determine the global methylation status and its association with tumor progression and platinum resistance[94]. Results revealed increased global hypermethylation in platinum-resistant samples compared to platinum-sensitive. In all, 5,844 differentially methylated probes (DMPs) and higher methylation levels were significantly associated with tumor progression, recurrence, platinum resistance and poor prognosis[94]. In another similar study, 749 DMPs were found between chemoresistant and chemosensitive tissue samples with 60% of them being hypermethylated in the resistant group[95].

However, in another study based on global methylation analysis of HGSOC cell lines, around 1251 out of the 1488 DMPs between chemosensitive and chemoresistant were hypomethylated in chemoresistant cells and were mostly located in non-coding regions of the genome[96]. These suggest the involvement of DNA methylation in ovarian cancer chemoresistance, and prognosis is complex and may depend on the molecular signature of the cancer cells.

Gene specific methylation has also been implicated in ovarian cancer chemoresistance. DNA mismatch repair protein (hMSH2) expression is lower in platinum-resistant EOC patients compared to platinum-sensitive patients and genome-wide methylation screening revealed hypermethylation at hMSH2 upstream region which was associated with poor survival[97]. Treating OVCA cells with DNMTi (5-aza-dC) resulted in increased expression of hMSH2 as well as enhanced cisplatin sensitivity, whereas knocking down hMSH2 resulted in reduced cisplatin-induced apoptosis[97]. DNA methylome and biological pathway analysis was performed in tissues from primary, recurrent, hypomethylating agents (guadecitabine) treated patient's cohort and ovarian surface epithelial cells.[98]. Differentially methylated genes were observed in all groups except the ovarian surface epithelial cells (control), Also, pathway analysis showed alteration in tumorigenesis, cell migration, invasion, EMT pathways and chemoresistance[98].

Significant hypomethylation at CpG sites of muscle segment homeobox gene MSX1 resulted in lower expression of MSXI in HGSOC patients resulting in disease progression, poor treatment response and short progression-free survival[99]. Further, overexpression of MSX1 in OVCA resistant cells increased their sensitization to cisplatin[99], suggesting the importance and relevance of gene specific methylation in ovarian cancer progression and treatment responses.

1.3. Gelsolin and OVCA Chemoresistance

i. Gelsolin Isoforms and Biological Functions

Gelsolin (GSN) is a member of the gelsolin superfamily that consists of seven other members including adseverin, adseverin D5, capG, villin, advillin, supervillin, and flightless[100]. GSN is a calcium-dependent actin-binding protein which is best known as regulators of actin skeleton and primarily responsible for cellular architecture and motility. This is achieved by regulating actin filament length, assembly of actin monomers through severing, capping and nucleating[101]. GSN

activity is regulated by two-signal mechanisms including intracellular Ca^{2+} concentration and pH[102], and phosphatidylinositol-4-5-bisphosphate (PIP_2)[103].

Depending on the kind of isoform, GSN has a molecular weight of 80-85kDa, and all isoforms are encoded by single gene on chromosome 9[104,105]. So far, three isoforms of GSN have been well characterized; cytoplasmic GSN (cGSN), secreted/plasma GSN (pGSN), and gelsolin-3, that are formed by alternative splicing and different transcriptional initiation sites[106] **(Fig. 3)**. cGSN and pGSN are two well defined isoforms involved in carcinogenesis which differ from one another by the presence of 24-amino acids signalling peptide extension at the N-terminal region in pGSN. Also, a disulfide bond between Cys188 and Cys201 residues enhances pGSN stability in the extracellular environment[104,107]. Gelsolin-3 is a recently discovered cytoplasmic GSN isoform, produced by oligodendrocytes, and is formed by alternative splicing of GSN gene. Gelsolin-3 also differs from the other isoforms by 11 additional residues at its N-terminus region[108].

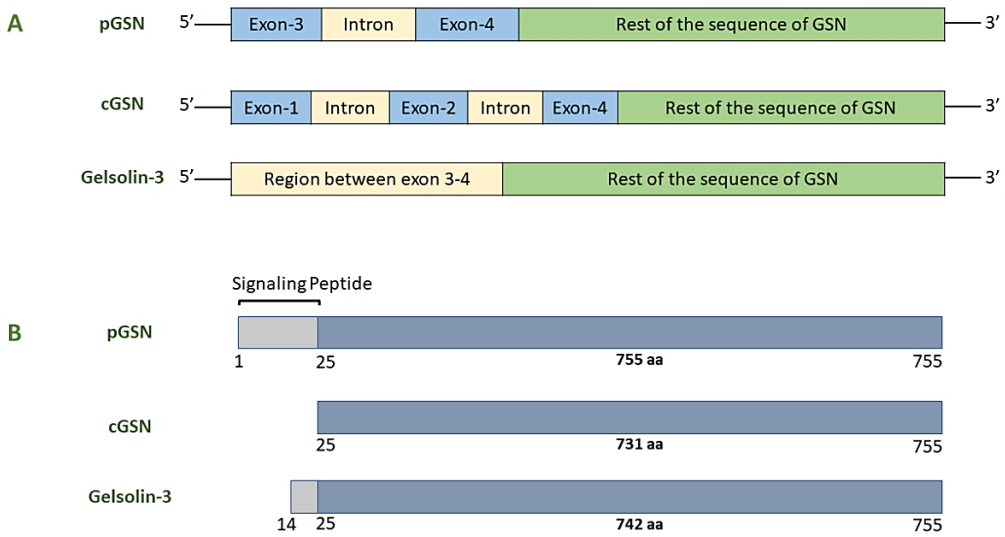

Figure 3. Genomic context and structure of GSN isoforms. (A) The pGSN, cGSN, and Gelsolin-3 are three well known isoforms of GSN, which are encoded by single gene and formed by alternative splicing and different transcriptional sites. Three isoforms are characterized by arrangement of 5`-end; pGSN 5`-end is made up of exon 3-intron-exon 4 while exon 3 make up untranslated region (UTR) and codes for signaling peptide, cGSN 5`-end made up of exon 1- intron- exon 2 – intron – exon 4, while exons 1 and 2 make UTR, 5`-end of Gelsolin-3 is made up of region between exon 3 and 4 which also make its UTR **(B)** All three isoforms have similar 733 amino acids, pGSN and Gelsolin-3 have extra 24 and 11 extra amino acids at N-terminus, respectively, which differentiate them from cGSN.

cGSN is intracellularly located and plays a key role in cytoskeletal remodeling and cell motility because of its ability to sever, cap/uncap, and nucleate actin filaments[109]. cGSN is a substrate for caspase-3 and upon being cleaved, it disassembles the cytoskeleton membrane and initiates apoptosis[100]. cGSN also safeguards mitochondrial integrity by inhibiting mitochondrial membrane loss[110]. cGSN has also been implicated in the regulation of chemosensitivity in HGSOC where its expression was significantly associated with tumor progression, progression-free and overall survival[55].

pGSN on the other hand is mainly secreted into the extracellular space with a smaller amount trapped in the cytoplasm of cells. The average concentration in circulation is estimated to be ~200-300µg/ml, and is mainly synthesized in the cardiac, skeletal, and smooth muscles instead of the liver, which is the major producer of mostly secreted proteins[104]. pGSN plays an important role of an extracellular actin scavenger in preventing actin toxicity and has also been implicated in various inflammatory diseases, bacterial and viral infections, malignancies and injuries[104,110].

Although the specific function of gelsolin-3 is not fully understood, it is proposed that gelsolin-3 plays a role in myelin remodeling and the central nervous system development[111].

ii. pGSN and OVCA Chemoresistance

Recently, pGSN has gained much attention because of its role in OVCA chemoresistance as well as in other malignancies[56,112–115]. In our previous findings, we have identified that pGSN is secreted via exosomes which activates the α5β1 integrins / FAK (focal adhesion kinase) / Akt / HIF-1α (hypoxia-inducible factor-1) axis resulting in increased endogenous production of pGSN in an autocrine and paracrine manner. Also, Increased pGSN expression protects ovarian cancer cells against cisplatin-induced apoptosis[56] **(Fig. 4)**. Chemoresistant patients express higher tumor levels of pGSN compared with their sensitive counterparts leading to poor overall survival. Additionally, pGSN activates the NRF2 pathway in ovarian cancer cells leading to increased production of GSH and other anti-oxidant factors, a process that deactivates cisplatin and inhibit cell death[56].

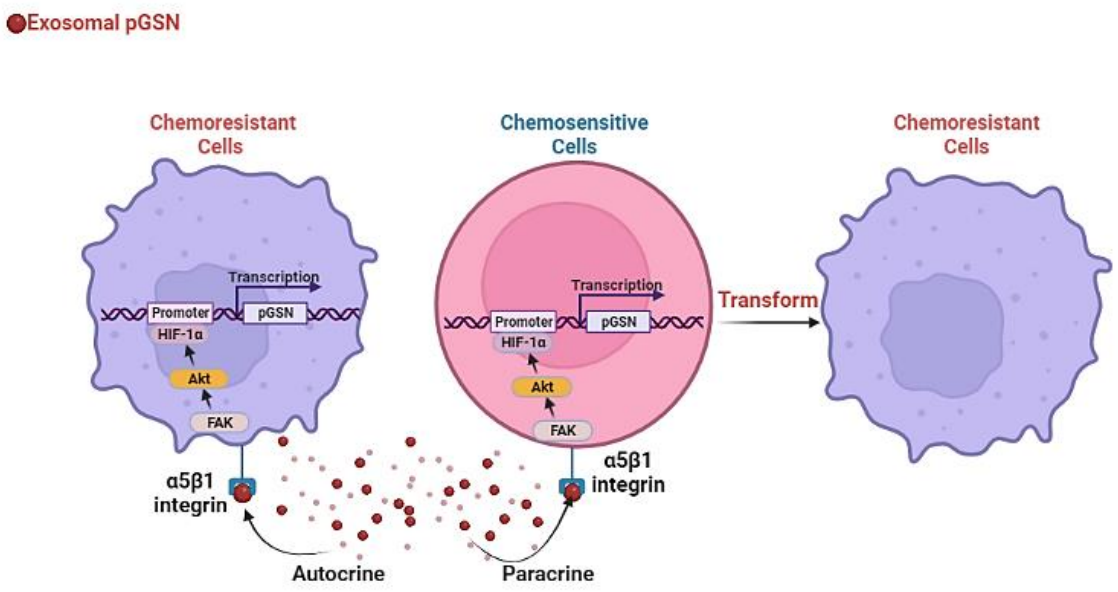

Figure 4. **Autocrine and paracrine mechanism of exosomal pGSN in OVCA chemoresistance.** Exosomal pGSN secreted from chemoresistant OVCA cells confers chemoresistance to otherwise sensitive OVCA cells by upregulating pGSN expression via α5β1 integrins/FAK/Akt/HIF-1α axis (paracrine action). Exosomal pGSN also auto-upregulate its own expression in chemoresistant OVCA cells via α5β1 integrins/FAK/Akt/HIF-1α axis (autocrine action). (Figure modified from[116] and designed using BioRender)

In chemosensitive OVCA cells, cisplatin significantly decreases Fas-associated death domain-like interleukin-1beta-converting enzyme (FLICE)-like inhibitory protein (FLIP) content by facilitating its interaction with itchy E3 ubiquitin protein ligase (Itch) and p53 resulting in FLIP ubiquitination and proteasomal degradation. This activates downstream cascades of caspase-8 and caspase-3 cleavage and induces apoptosis[116,117]. It has been proposed that GSN forms a stable complex with FLIP-Itch which is dissociated by cisplatin action in chemosensitive OVCA cells, resulting in the activation of caspase-mediated GSN cleavage and promotion of apoptosis. However, in chemoresistant OVCA cells high GSN expression prevents cisplatin from attenuating GSN-FLIP-Itch complex and hence inhibiting the activation of caspase cascade and apoptosis[55].

Tumor-infiltrating immune cells are crucial in the tumor microenvironment during tumorigenesis and treatment responses. Although increased tumor infiltration of CD8+ T cells

and macrophages provides survival benefits to ovarian cancer patients, their responses are inhibited by increased pGSN in these patients. This pGSN-mediated inhibitory effect results in shortened patient survival as well as poor treatment responsiveness. Mechanistically, pGSN is believed to induce caspase-3 activation and trigger cell death in the immune cells. Additionally, pGSN inhibits the production of anti-tumor factors such as interferon gamma (IFNγ), Tumor-necrosis factor α (TNFα), granzyme β and nitric oxide, an action that disarms the adaptive and innate immune system[118,119]. Aside killing immune cells, pGSN also preferentially polarizes CD4 helper T cells to Th2 cells, thus contributing to a tumor thriving environment. All these finding support the notion that pGSN is indispensable in OVCA progression, treatment response and chemoresistance.

Secretory pGSN has also proven to be a potential marker for predicting early-stage disease, residual disease, and treatment responses in ovarian cancer patients. Their clinical utility is further enhanced when secreted via exosomes and also outperforms the conventional biomarker, CA125[120,121]. Although the diagnostic and therapeutic properties of pGSN have been investigated, we have yet to determine whether the differential levels of pGSN between chemoresistant and chemosensitive patients are because of epigenetic regulation.

iii. Epigenetic Regulation of Gelsolin Gene

To date, no major naturally occurring mutation, deletion, or rearrangement in the gelsolin gene has been identified that regulate its role in the context of cancer. However, a number of studies have reported epigenetic regulation of gelsolin expression including DNA methylation, histone and miRNA modification[122–128]. It has been reported that hypermethylation at CpG island of the GSN gene occurs in human breast cancer cells, a process which was associated with decreased GSN expression compared with human epithelial mammary cells. An increase in GSN mRNA and protein expression was observed after treating human BC cells with 5-azacytidine; 5-aza (DNMTi) and trichostatin A; TSA (HDACi). However, hyperacetylation effect by TSA on GSN upregulation was more significant compared to the hypomethylation effect by 5-aza[122]. Similar results were observed when ovarian cancer cells expressing low GSN expression compared to normal ovarian tissues were treated with 5-aza and TSA[123]. While investigating mechanism behind anti-proliferative effect of HDACi in human glioma cells, high expression of p21 (cell cycle inhibitor) and GSN was observed after treatment with two different kinds of

HDACi; TSA and sodium butyrate (NaBT), suggesting GSN expression might be regulated by histone acetylation in human glioma cells[125]. In a recent study with gastric cancer cells, GSN gene silencing was found associated with increased DNA methylation in its promoter region induced by DNMT1 expression. Tumor associated macrophages (TAMs) stimulated the DNMT1 expression in gastric cancer cells by secreting CCL5 chemokines, which is believed to further activate the JAK2/STAT3 pathways[127]. In addition to DNA methylation and histone modification, pGSN has recently been shown to be negatively regulated by miR-654-5p and miR-450b-5p in glioblastoma cells. Inhibition of miR-654-5p and miR-450b-5p resulted in increased expression GSN which decreased cell viability[128]. Taken together, it is evident that GSN is epigenetically regulated in human cancers either by the action of DNA methylation, histone acetylation or miRNA action. These findings suggest that GSN expression could be epigenetically regulated in human gynecological cancers[124]. Hence, new studies on epigenetic regulation of pGSN could provide a better understanding of the control of its expression and function in OVCA chemoresistance.

1.4. Ten Eleven Translocation Enzyme and Chemoresistance

i. Biological Function and Isoforms

The three members of the TET family regulate DNA demethylation and maintain methylation patterns in the genome. These enzymes are α-ketoglutarate (α-KG)/Fe (II) dioxygenases that catalyze the DNA demethylation by causing hydroxylation of 5mC to generate 5hmC. 5hmC is further oxidized to 5-formylcytosine (5fC) and 5-carboxycytosine (5caC), rapidly eradicated by TDG and restored back to unmethylated cytosine by base excision repair mechanisms[129–133]. TETs also regulate other metabolic reactions including collagen synthesis, response to hypoxic condition, and catalysis of multiple oxidative reactions such as hydroxylation, epoxidation, epimerization and others in animals, plants and micrroganisms[134,135]. 5hmC is relatively abundant in embryonic stem cells (ESCs) whereas their level decreases after differentiation[136]. TET proteins are also abundant in transcription regulatory regions of neuronal cells, as well as, in the gene bodies and promotor regions, especially those rich in CpG dinucleotides[130,137]. The 5mC derivatives by TET enzymes assist in the transcriptional regulation of methylated genes by reducing the DNA-binding affinity of methyl cytosine binding protein (MeCP) and serve as a stable epigenetic mark[138–140].

Although all three isoforms of TET proteins possess the same cysteine rich C-terminal catalytic domain and a double-stranded β-helix fold, they are associated with different biological processes due to their cell specificity and differential expression[131,141]. TET1 and TET3 have an additional Cys-Xaa-Xaa-Cys (CXXC) domain at their N-terminus which facilitates their binding to DNA[142]. Multiple mutations in TET enzymes are reported in different solid and myeloid tumors where their altered expression and function promote aberrant DNA methylation[143]. TET1 and TET3 mutations are mostly reported in hematopoietic cancers whereas TET2 mutations are more common in myeloid disorders[144]. Further, low 5hmC levels are observed in several cancer types, suggesting that TET proteins are involved in cancer development and progression[145]. In OVCA, TET enzymes are found to be associated with tumor progression, metastasis and chemoresistance development[146–151]. TET1 inhibited cell proliferation and tumor growth in ovarian cancer by increasing 5hmC level and upregulating Ras association domain family member 5 (RASSF5) expression[146]. Reduced TET2 expression was found to be associated with poor prognosis of OVCA patients[148,149]. Further, TET3 overexpression suppresses ovarian cancer growth by blocking TGF-β1-induced EMT[150]. Although, there are growing evidence suggesting the role of TET enzymes in OVCA development, progression and chemoresistance, the underlying mechanisms still need to be explored to fully understand their role and to explore their potential as a therapeutic target.

ii. TET1 And Chemoresistance

TET1 is the most studied isoform of TETs family and primarily responsible for 5mC to 5hmC oxidation[90,152,153]. Recently, several biological functions regulated by TET1 have been identified in different malignancies. TET1 has been reported to have dual tumor-promoting and tumor-suppressing functions in cancer development, progression and treatment responses[153–155]. Also, a number of studies have highlighted TET1's role as a promising target to overcome chemoresistance in different cancers[151,156–159]. TET1 promotes the 5-Fluorouracil (5-FU) resistance in colorectal cancer cells via demethylating nuclear factor- erythroid 2-related factor 2 (Nrf2) promoter and upregulating its expression[156]. Increased expression of Nrf2 transcription factor enhances the expression of heme oxygenase-1 (HO-1), a Nrf2-regulated gene which has antiapoptotic and proangiogenic effects[156]. In OVCA cell model, TET is highly expressed in platinum resistant cells causing promoter de-methylation and upregulation of EMT marker,

Vimentin and inducing EMT in chemoresistant cells[151]. Decreased TET1 expression was observed in gemcitabine cholangiocarcinoma (CCA), and it was found to upregulate the expression of P-glycoproteins, a multi-drug resistant protein. Overexpression of TET1 sensitized the CCA cells to gemcitabine treatment. TET1-mediated hydroxymethylation was found to have role in gastric cancer chemoresistance development by upregulating chemoresistance-related protein; cellular retinoic acid binding protein 2 (CRABP2). CRABP2 promotes oxaliplatin resistance in gastric cancer cells by promoting cell proliferation and inhibiting apoptosis[159]. All these finding greatly support the notion that TET1 plays an indispensable role in the development of chemoresistance in different human cancer and can be a potential target for improving cancer treatment approaches.

2. RATIONALE

OVCA is the third most common gynaecological cancer and the leading cause of cancer related deaths in women[1]. Despite the advances in diagnosis and treatment approaches, the 5-year survival rate remains between 30-40% around the globe[7]. The first line of treatment for OVCA is a cytoreductive surgery combined with chemotherapeutic treatment of platinum and taxane-derivatives. About 70–80% of the patients after initially responding to platinum-chemotherapy relapses and become resistant to the treatment, causing over 90% of deaths[45,46]. Therefore, chemoresistance development has been a major challenge in ovarian cancer treatment.

DNA methylation is a well-known epigenetic mechanism associated with gene regulation in cancer development, progression, recurrence and chemoresistance. The role of altered DNA methylation, both at specific gene and the global level, in the ovarian cancer chemoresistance development has extensively been explored.

Previously, we have identified that pGSN is highly expressed in OVCA chemoresistant cells compared to its chemosensitive counterparts. Also, Increased pGSN expression protects ovarian cancer cells against cisplatin-induced apoptosis[56]. However, the mechanism behind pGSN differential expression between the OVCA chemosensitive and chemoresistant cells is poorly understood. Multiple studies have reported that GSN expression could be epigenetically regulated in human gynecological cancers[124]. Hence, new studies on epigenetic regulation of pGSN could provide a better understanding of the control of its expression and function in OVCA chemoresistance. Recently, increasing evidence has revealed the role of TET1 in hypomethylating candidate genes that leads to cancer development and chemoresistance[151–156]

To the best of our knowledge, methylation status of pGSN in OVCA and its association with clinical outcomes has not been reported to date. Further, whether TET1 has any role in the regulation of pGSN in OVCA chemoresistance remains to be investigated.

3. HYPOTHESIS AND OBJECTIVES

3.1. Hypothesis

We hypothesize that pGSN overexpression in chemoresistant OVCA cells is due to the hypomethylation at its promoter region by increased TET1 expression **(Fig. 5)**.

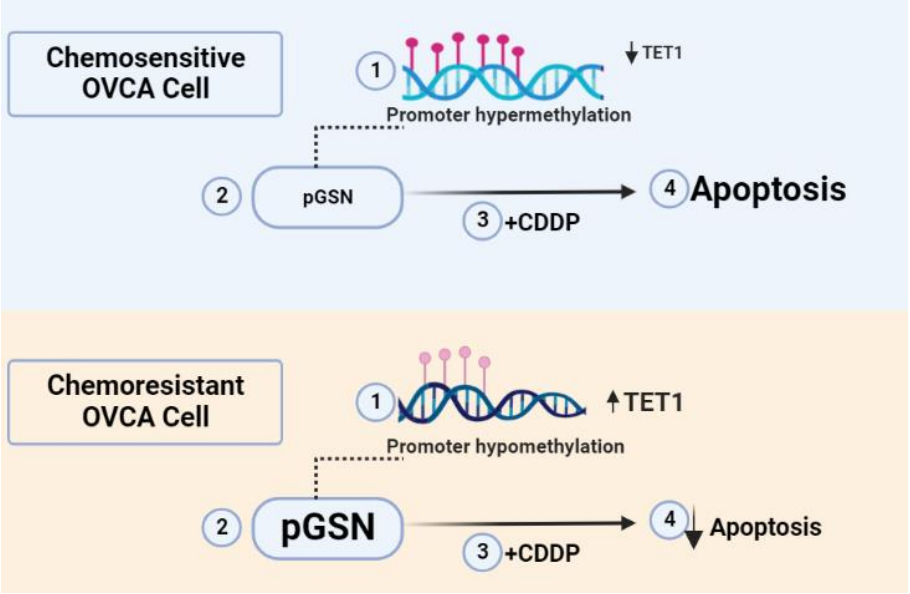

Figure 5. Hypothetical model illustrating TET1 regulating pGSN promoter methylation status, its expression and chemo-responsiveness of OVCA cells.

3.2. Objective

The overall objective of this study is to investigate whether the differential pGSN expression in OVCA cells is due to pGSN promoter methylation.

The specific objectives of this study are:

1. To identify the association between pGSN and TET1 expression as well as OVCA cell chemo-responsiveness.
2. To examine whether DNA methylation is important in the regulation of pGSN expression and chemo-responsiveness in OVCA cell lines.
3. To investigate the role of TET1 in pGSN-mediated chemoresistance in OVCA cells.

4. MATERIALS AND METHODS

i. TCGA Dataset Analyses

Publicly available ovarian cancer dataset (www.kmplot.com) were interrogated to identify the association between TET1 (affymetrix Id: 228904_at) expression and OVCA patients' survival. Patients' information was stratified based on histological subtype (serous), surgical outcome (optimal/suboptimal debulking), and chemotherapy treatment (platinum)[160]. Further, all tumor stages, grades and p53 statuses were included in the analysis. Progression-free survival; PFS (the time from surgical treatment to further disease progression in the patients) and overall survivals; OS (the time from surgical treatment to the time of death) of OVCA patients were correlated with TET1 expression using Kaplan Meier plotter. The log-rank test was used for statistical parameter calculation and graph plot was used for visualization[160]. GEPIA (www.gepia.cancer-pku.cn) public dataset was used to demonstrate the correlation between GSN and TET1 gene expression in ovarian cancer tissues. Statistical parameters were calculated using spearman correlation coefficient, non-log scale for calculation and log-scale axis for visualization[161].

ii. Reagents

Cis-diaminedichloroplatinum (CDDP, Cat# P4394), 5-azacytidine (Cat# A2385), Bobcat339 (Cat # SML2611), anti-pGSN antibody (anti-gelsolin antibody, mouse monoclonal, Cat # SAB4200750) and dimethyl sulfoxide (DMSO, Cat # D8418) were purchased from Sigma-Aldrich (Oakville, Canada). TriFECTa® RNAi kit (hs.Ri.TET1.13) was purchased from Integrated DNA Technology (Iowa, USA) for gene silencing assays, whereas TET1 cDNA (TET1 cDNA ORF Clone, Human, C-GFPSpark® tag, Cat # HG19726-ACG) and empty control vector (pCMV3-C-GFPSpark, Cat# CV026) were purchased from SinoBiological (South Carolina, USA). Anti-TET1 antibodies were purchased from Abcam (Toronto, Canada); Cat# ab272900, ab272901, ab191698, Thermofisher scientific (Nepean, Canada); Cat# PA5-85489, and Active motif (California, USA); Cat# 91171. Primers (TaqMan gene expression assays; TET1 (Cat # 4331182, Assay ID: Hs00286756_m1), pGSN (Cat # 4331182, Assay ID: Hs00609272_m1), and ATCB (Cat# 4333762T)), cDNA synthesis kit (High-Capacity cDNA reverse transcription kit, Cat # 4368814) and Hoechst 33258 (Cat# 94403) stain were bought from Thermofisher scientific (Nepean, Canada). Extraction kits for RNA (RNeasy mini kit, Cat#

74104) and DNA (AllPrep DNA/RNA mini kit, Cat#80284, DNeasy blood & tissue kit, Cat # 69504) were from Qiagen (Toronto, Canada). TET activity kit (Epigenase 5mC-Hydroxylase TET activity/Inhibition assay kit, Cat# P-3086) was purchased from EpigenTek Group Inc (New York, USA). TET1 (Cat#31417), TET2 (Cat#31418), and TET3 (Cat#31421) recombinant proteins were from Active motif, Inc, (California, USA).

iii. Cell Lines and Cell Culture

Chemosensitive and chemoresistant OVCA cell lines of endometrioid and HGS histologic subtypes were used in this study. The characteristics of the cells are as follows: endometrioid cells [A2780S (p53 wildtype- sensitive) A2780CP (p53 mutant; V127F, R260S-resistant)], and HGS [TOV3133G (nonsense-sensitive), TOV3133R (p53 mutant; Gln192Ter-resistant)[162–166]. Endometrioid and HGS cell lines were cultured in RPMI-1640 and OSE media respectively, with 10% fetal bovine serum (FBS) at 37ºC in humidified 5% CO_2 incubator. For cisplatin treatment, 10µM of cisplatin was added to the cells in the absence of serum[116,117,167].

iv. RNA interference

Chemoresistant cells were transfected with three different siRNAs targeting TET1 (hs.Ri. TET1.13.1,2,3; Integrated DNA Technology (IDT); 0-20 nM) and negative scrambled control siRNA (Dicer Substrate (DS) Negative Control 1; IDT) with lipofectamine RNAiMax transfection reagent for 48 hours followed by CDDP treatment (0-10 µM; 24 h). Using multiple siRNA helps to rule out off target effects of gene silencing[116,117,167,168]. Transfection efficiency was confirmed by using positive control (HPRT-S1 DS positive duplex control; IDT) and fluorescently labeled transfection control (TYE^{TM} 563 DS transfection control: IDT). Successful knockdown was confirmed by qPCR as per TaqMan gene expression assay protocol.

v. Transient Transfection

Chemosensitive cells were transiently transfected with TET1 cDNA (TET1 cDNA ORF clone, Human, SinoBiological; 0-2 µg; 24 h) and an empty vector control (pCMV3-C-GFPSpark® vector, SinoBiological; 0-2 µg; 24 h) using lipofectamine 2000 transfection reagent, followed by CDDP treatment (0-10 µM; 24 h)[116,117,167]. Successful knockdown was confirmed by qPCR as per TaqMan gene expression assay protocol.

vi. Pharmacological Inhibition of DNMTs and TETs

Cells were treated with 0-10 μM 5-Azacytidine (DNMTi) and TETs inhibitors (BobCat339; 0-100 μM) once a day for two days followed by 0-10 μM cisplatin (24 h). After cisplatin treatment, cells were harvested for subsequent analysis.

vii. RNA Extraction and Quantification

Total RNA was extracted from cells using RNeasy mini kit (Qiagen) according to the manufacturer's protocol. RNA concentration and purity were confirmed using Nanodrop spectrophotometer. cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Thermofisher scientific) according to manufacturer's protocol. Quantitative polymerase chain reaction (qPCR) was performed using TaqMan gene expression assay system on Applied Biosystems 7500 Fast Real-Time PCR System. mRNA levels of target genes were quantified relative to ACTB mRNA level. All reactions and experiments were carried in triplicates.

viii. Protein Extraction and Quantification

Cells were collected after trypsin treatment, washed with phosphate-buffered saline (PBS), centrifuged (13,000 g for 5 minutes) and suspended in a complete lysis buffer (cOmplete, Mini protease inhibitor cocktail tablets dissolved in cOmplete lysis-M reagent-Roche). Samples were sonicated (30% amplitude for 30s), and the cell lysate collected after centrifugation for 30 minutes (13,000 RPM at 4ºC). Protein concentration was measured using DC (Detergent compatible) protein assay (Bio-Rad). Equal amounts of protein were loaded into 10% SDS-PAGE gel for electrophoresis and transferred to nitrocellulose membrane. After protein transfer, membranes were blocked with 5% blotto (skim milk in TBST buffer; 1 h) followed by overnight incubation at 4ºC with primary antibodies (1:1000) and ultimately incubation with respective horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000; 1 h; RT)[55,116,117]. Both primary and secondary antibodies were prepared in 5% blotto. After secondary antibody incubation, membranes were washed with TBST (3x; 15 mins each). The protein bands were visualized using Enhanced chemiluminescent (ECL) kit (Thermofisher scientific) on ChemiDoc Imaging system (BioRad) and analyzed by measuring the densities of protein bands using Image J software.

ix. DNA Extraction and Quantification

Genomic DNA was extracted from cells using AllPrep DNA/RNA mini kit and DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. DNA concentration and purity were quantified using Nanodrop spectrophotometer.

x. Global DNA Methylation Analysis

The extracted genomic DNA (500 ng) was bisulfite converted using EZ DNA methylation kit (Zymo Research) following the manufacturer's protocol for Infinium HumanMethylation450 BeadChip assay. Global DNA methylation was performed with 450K DNA methylome-wide array assay[169].

xi. Quantitative DNA Methylation Analysis

Extracted DNA from OVCA cell lines was bisulfite treated and amplified with PCR followed by purification of PCR products with shrimp alkaline phosphatase (SAP) treatment. Th genomic region of interest for pGSN spanned 602 base pairs (GRCH37-hg19: chr9:124061710-12406231269, CpG units) and included coverage over the reported CpG island as well as CpG sites in the promoter region. A single-stranded RNA copy of the region of interest was generated by *in-vitro* transcription using reverse pGSN PCR primers tagged with the T7 recognition sequence. The resulting transcript was base-specifically (U-specific) cleaved followed by MALDI-TOF mass spectrometry quantification of methylation ratio and results were analyzed with EpiTYPER software[170]. The region of interest for pGSN was analyzed in 2 fragments spanning approximately 300 base pairs each. Highly methylated and low methylated human control DNA were also included in the assessment. Methylation data was screened for unreliable methylation ratios having low/high mass outside the detection limit of analyzer and more than one silent peak.

xii. TET Enzymatic Activity Assay

Enzymatic activity of TETs was quantified using Epigenase 5mC-Hydroxylase TET Activity/Inhibition Assay Kit (Colorimetric) as per manufacturer's protocol.

xiii. Drug Sensitivity Assay

Cells were seeded in 96-well plate and treated with 10 µM CDDP for 24 hours. The cell viability was assessed using cell counting kit-8 (CCK8) with an optical density of 450 nm.

xiv. Apoptosis Analysis

Morphological assessment of CDDP-induced apoptosis was performed using Hoechst 33258 nuclear stain and visualized with fluorescence microscopy (ZOE fluorescent cell imager-BioRad). A minimum of 400-500 cells with apoptotic morphology were counted from random fields using blinded approach to avoid experimental bias. Apoptotic cells were expressed as the percentage of total cells.

xv. Statistical Analysis

All the experiments were performed in three independent replicates and results were presented as mean ± SEM. Two-way or three-way ANOVA were performed for statistical purposes and Tukey *post-hoc* was used for multiple comparisons. Differences were considered significant at $p < 0.05$.

5. RESULTS

i. TET1 expression is associated with poor prognosis of ovarian cancer patients.

Publicly available ovarian cancer dataset (www.kmplot.com) were interrogated to identify the association between TET1 (affymetrix Id: 228904_at) expression and OVCA patients' survival. Patients' information was stratified based on histological subtype (serous), surgical outcome (optimal/suboptimal debulking), and chemotherapy treatment (platinum). Further, all tumor stages, grades and p53 statuses were included in the analysis. Progression-free survival (PFS) and overall survivals (OS) of OVCA patients were correlated with TET1 expression using Kaplan Meier plotter. The log-rank test was used for statistical parameter calculation and graph plot was used for visualization. In serous carcinoma patients who received platinum-based chemotherapy treatment, high TET1 expression was significantly (p=0.00045) associated with shortened PFS (14 months) compared with patients with lower TET1 expression (18.07 months). Similarly, high TET1 expression was also significantly (p=0.0031) associated with low OS (37.93 months) compared with patients with lower TET1 expression (48 months) **(Fig. 6A)**.

Previously, it's been demonstrated that GSN over-expression significantly correlate with poor survival in ovarian cancer patients[56] hence, we used GEPIA (www.gepia.cancer-pku.cn) public dataset to demonstrate if there is any correlation between GSN and TET1 gene expression in ovarian cancer tissues. Statistical parameters were calculated using spearman correlation coefficient, non-log scale for calculation and log-scale axis for visualization. We found a significant positive correlation between TET1 and GSN expression in human OVCA tissues (p=0.00044) **(Fig. 6B)**.

Information extracted from the public database provided us with the rationale to investigate the role of TET1 in the regulation of pGSN expression and chemoresistance development in OVCA cells.

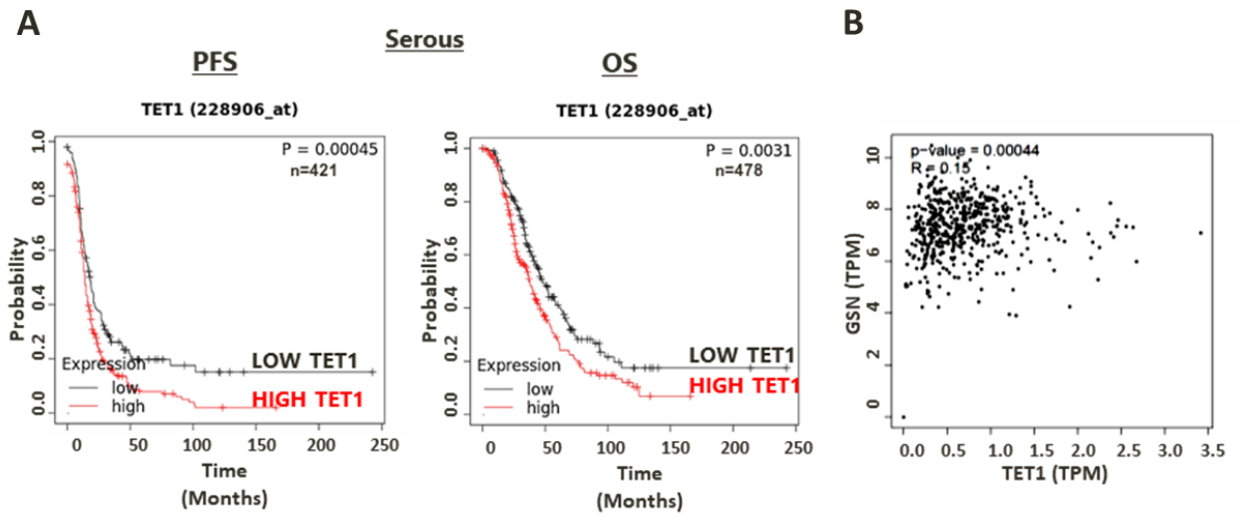

Figure 6. High TET1 expression significantly correlate with increased recurrence and shortened survival of OVCA patients (A) OVCA public data sets (Kaplan-Meier Plotter) was analyzed for TET1 correlation with patient survival and **(B)** GEPIA was used to demonstrate the correlation between GSN and TET1 gene expression in ovarian cancer tissues.

ii. pGSN is highly expressed in chemoresistant OVCA cells compared to their sensitive counterpart.

Firstly, we validated previous findings from our lab where we reported that OVCA chemoresistant cells express high pGSN compared to chemosensitive cells; a phenomenon that is also associated with decreased chemoresponsiveness of chemoresistant OVCA cells[56].

Endometrioid (sensitive, A2780s; resistant, A2780cp) and HGS (sensitive, TOV3133G; resistant, TOV3133R) cell lines were cultured in RPMI-1640 and OSE media respectively, with 10% fetal bovine serum (FBS) at 37ºC in humidified 5% CO_2 incubator. After achieving confluency, cells were seeded into 6 wells and 96 wells plates for cisplatin (cis-diamminedichloroplatinum; CDDP) treatment (10µM) and 0.1% dimethyl sulfoxide (DMSO) as a control for 12-48 hours. Cells were harvested for endpoint analyses, including pGSN mRNA abundance (qPCR) and protein content (Western blot) along with cell viability (CCK-8 assay) and apoptosis assay (Hoechst staining).

Significant increase in apoptosis ($p<0.0001$) and reduction in cell viability ($p<0.0001$) were observed after CDDP treatment in chemosensitive cells compared to chemoresistant cells **(Fig. 7)**. Significantly higher pGSN mRNA abundance [A2780cp ($p=0.0089$), TOV3133R ($p=0.0002$)] and protein content [A2780cp ($p<0.0001$), TOV3133R ($p=0.0039$)] were observed in resistant cells compared to their sensitive counterparts. CDDP treatment significantly decreased pGSN mRNA ($p=0.0049$) and protein contents ($p=0.0112$) in sensitive cells (A2780s) but not in resistant cells. **(Fig. 8)**.

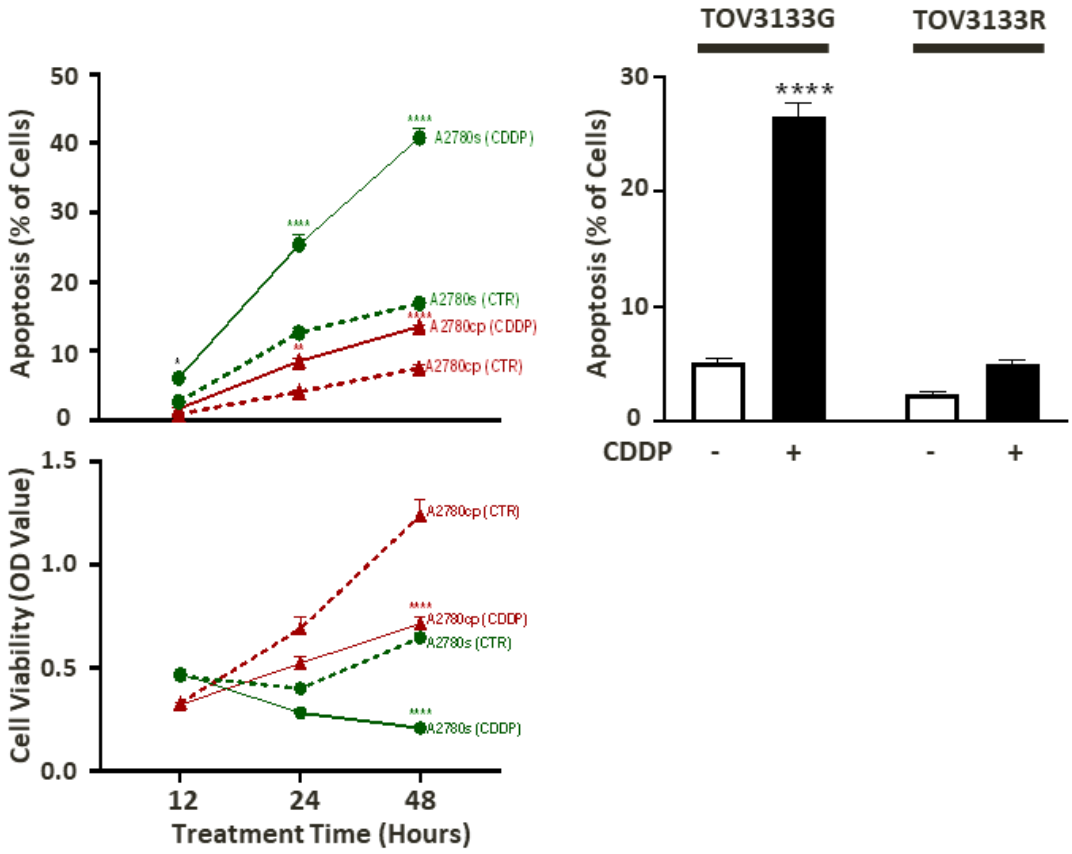

Figure 7. Significantly higher chemoresponsiveness observed in sensitive cells (endometrioid, A2780s; HGS, TOV3133G) compared to OVCA resistant cells (endometrioid, A2780cp; HGS, TOV3133R). OVCA cells were treated with or without CDDP (10 µM; 24 h). Apoptosis was morphologically determined by Hoechst 33258 nuclear staining and cell viability examined using CCK8 assay. (mean ± SEM; n=3). Differences between all the groups were evaluated using two-way and three-way ANOVA followed by Tukey's multiple comparison test; *p*<0.05, p**<0.01, p***<0.001, p****<0.0001 compared to respective CTR group.*

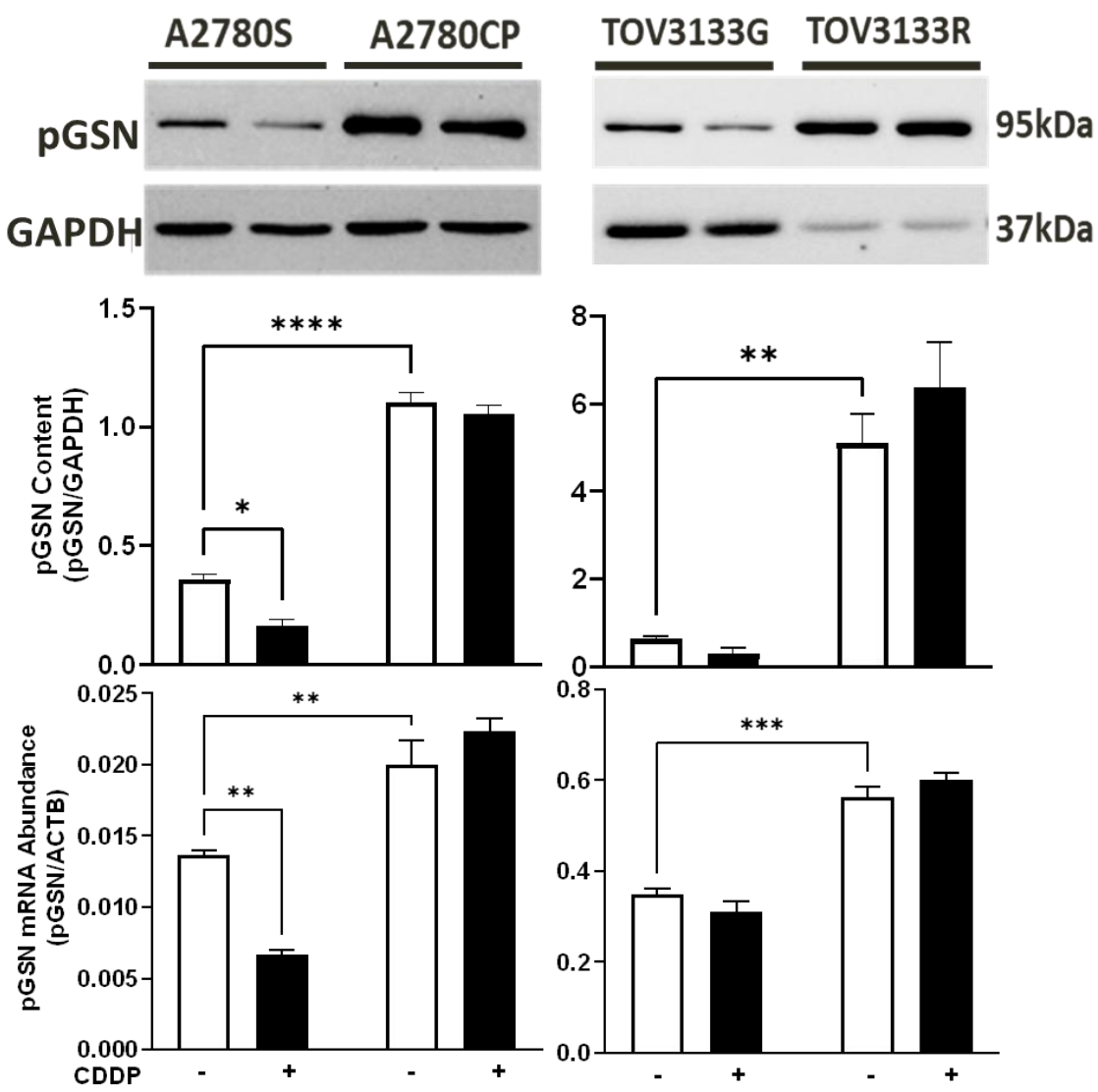

Figure 8. Significantly higher pGSN mRNA and protein contents observed in OVCA resistant cells (endometrioid, A2780cp; HGS, TOV3133R) compared to sensitive cells (endometrioid, A2780s; HGS, TOV3133G). OVCA cells were treated with or without CDDP (10 µM; 24 h). pGSN mRNA content relative to ACTB (loading control) was assessed by qPCR. pGSN and GAPDH (loading control) protein contents were assessed by Western blotting (mean ± SEM; n=3). Differences between all the groups were evaluated using two-way ANOVA followed by Tukey's multiple comparison test; *p*<0.05, p**<0.01, p***<0.001, p****<0.0001*.

iii. TET1 is differentially expressed between chemosensitive and chemoresistant OVCA cells.

After validating differential expression of pGSN in OVCA chemosensitive and chemoresistant cells, we further examined TET1 expression using similar experimental design where chemosensitive and resistant cells were treated with CDDP (10 µM; 24 h). Cells were harvested and processed to measure TET1 mRNA content (qPCR), and protein content (Western blot).

In endometrioid OVCA cells, significantly higher TET1 mRNA content was observed in chemosensitive cells (A2780s) compared to resistant counterpart (A2780cp) ($p= 0.0052$). Additionally, CDDP significantly decreased TET1 mRNA content in sensitive cells but not in resistant cells ($p=0.0008$). However, contrary to mRNA levels, high TET1 protein content was observed in chemoresistant cells compared to their chemosensitive counterparts. Western blot analysis revealed bands at two different positions (between ~70kDA- 130kDa). The basal levels of both the upper and lower bands of TET1 are higher in resistant cells compared to chemosensitive cells. The upper TET1 band is decreased in both cells after CDDP treatment; however, the lower band is unaffected in the resistant but upregulated in the sensitive cells after CDDP treatment. Although different antibodies against TET1 were used to analyze its protein content, we were unsuccessful in getting bands at the expected position (~235kDa)**.**

In HGS cells, however, we observed significantly higher TET1 mRNA content in resistant cells (TOV3133R) compared to sensitive counterpart (TOV3133G) ($p<0.0001$). Although a decrease in TET1 was observed in the sensitive cells after CDDP treatment, this change was not significant.

These findings suggests that TET1 expression might be histological subtype dependent in OVCA cells. Also, TET1 stability might be stronger in resistant cells compared to their sensitive counterparts, explaining why CDDP could only decrease TET1 in the sensitive cells but not the resistant cells **(Fig. 9)**.

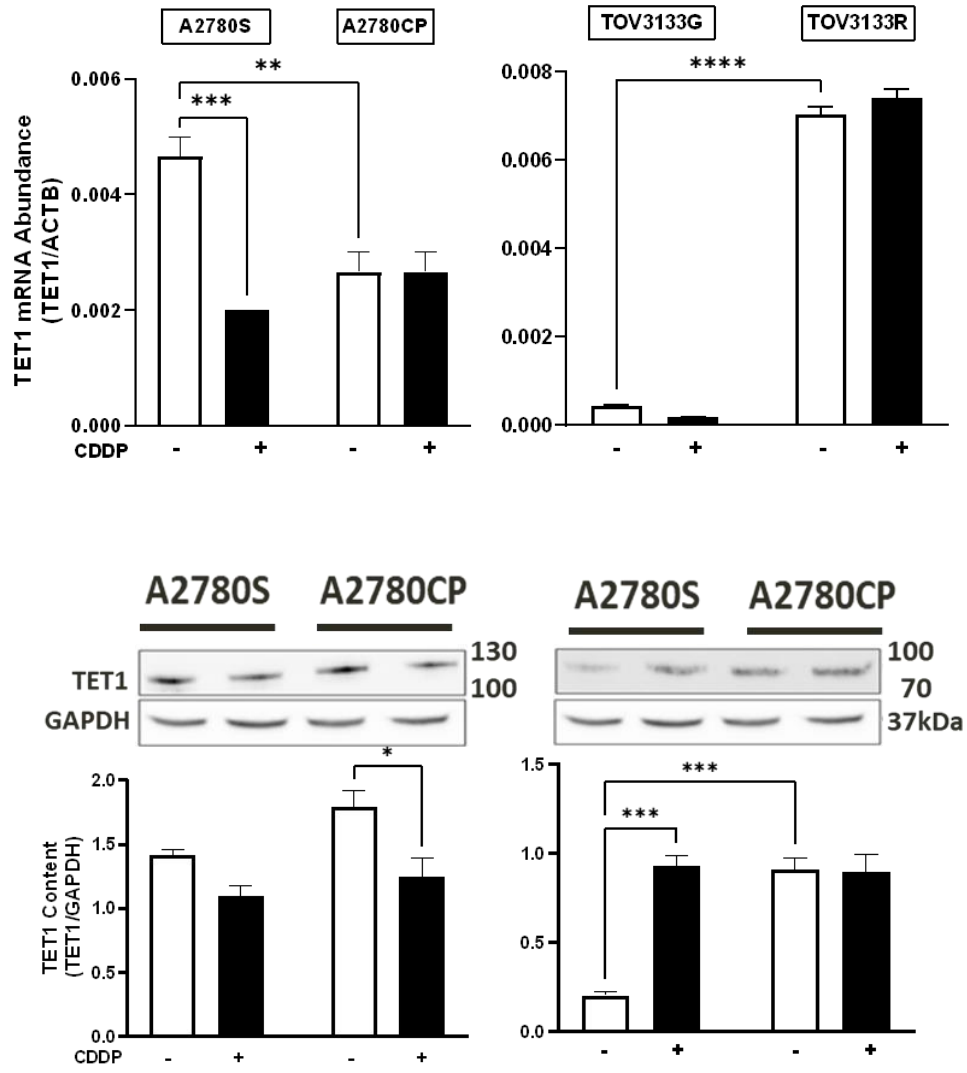

Figure 9. TET1 is differentially expressed between chemosensitive and chemoresistant OVCA cells. OVCA cells were treated with or without CDDP (10 µM; 24 h). TET1 mRNA content relative to ACTB (loading control) was assessed by qPCR. TET1 and GAPDH (loading control) protein contents were assessed by Western blotting (mean ± SEM; n=3). Differences between all the groups were evaluated using two-way ANOVA followed by Tukey's multiple comparison test; *p*<0.05, p**<0.01, p***<0.001, p****<0.0001*.

Cell lysates after CDDP treatment were also used to quantify the enzymatic activity of TETs by measuring the hydroxylase activity as per manufacturer's protocol using epigenase 5mC-hydroxylase TET activity assay kit. We did not observe any significant differences in the TETs activity among sensitive (A2780s, TOV3133G) and resistant (A2780cp, TOV3133R) OVCA cells with and without CDDP treatment **(Fig. 10)**.

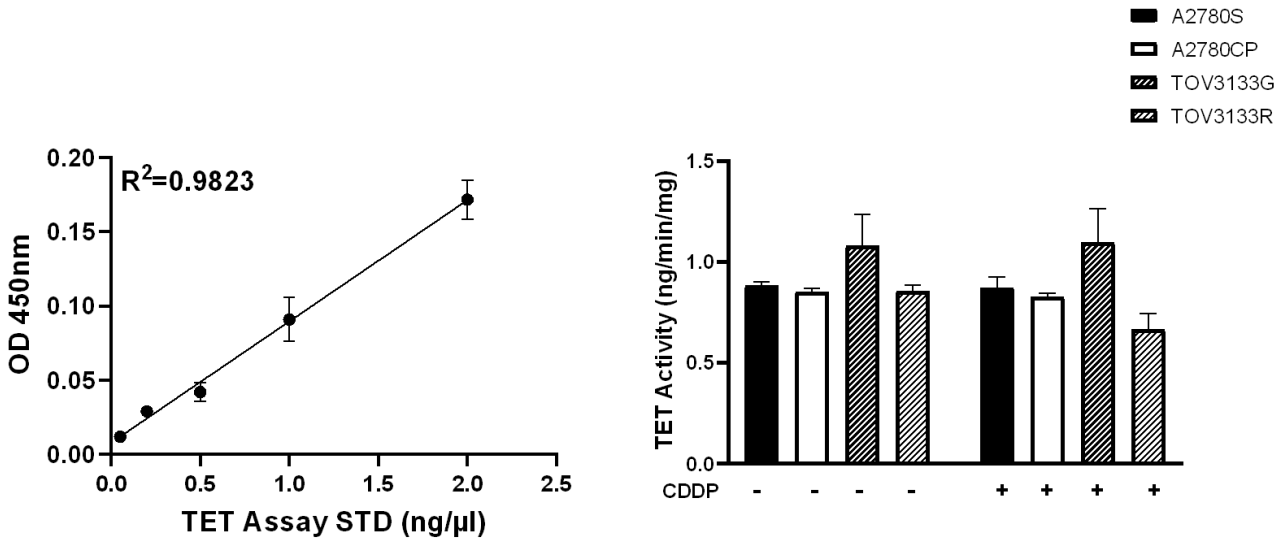

Figure 10. No significant differences in the TETs activity observed among all OVCA cell types. OVCA cells were treated with or without CDDP (10 µM; 24 h). TET activity was assessed using epigenase 5mC-hydroxylase TET activity assay kit. Standard curve was generated for TET activity assay using TET assay standard provided with the kit (mean ± SEM; n=3). Differences between all the groups were evaluated using two-way ANOVA followed by Tukey's multiple comparison test; *p*<0.05, p**<0.01, p***<0.001, p****<0.0001, A2780s vs A2780cp and TOV3133G vs TOV3133R.*

iv. Differential methylation level between chemosensitive and chemoresistant OVCA cells.

The role of altered DNA methylation, both at specific genes and the global level, in ovarian cancer chemoresistance development has extensively been explored[94–99]. To confirm whether DNA methylation is related with the chemoresponsiveness of OVCA cells, we analyzed the global DNA methylation status in chemosensitive and chemoresistant OVCA cells. Genomic DNA was extracted from chemosensitive and chemoresistant OVCA cell lines of different histological subtypes **(Table 2)** and sent to the International Agency for Research on Cancer (IARC) lab (a collaboration through Dr. Chantal Matar, Faculty of Medicine, University of Ottawa). The extracted DNA (500 ng) was bisulfite-converted using EZ DNA methylation kit and global DNA methylation analysis based on Infinium HumanMethylation450 BeadChip array was performed.

Comparison of DNA methylome from chemoresistant and chemosensitive cells revealed a total of 366 differentially methylated regions (DMRs) with cut-off criteria; 5% methylation difference and adjusted p value <0.05. 53.3% of DMRs were hypomethylated in resistant cells compared to sensitive cells and 46.7% DMRs were hypermethylated in resistant cells **(Fig. 11).** These findings suggest that methylation; specifically, hypomethylation plays a key role in OVCA chemoresistance.

Table 2. Information about OVCA cell lines used for the global methylation analyses.

#	Cell lines	Tumor Origin	Chemo-responsiveness	TP53 Status
1	A2780s	Endometroid adenocarcinoma	Sensitive	Wild type
2	A2780cp	Endometroid adenocarcinoma	Resistant	Mutant (V127F, R260S)
3	TOV112D	Endometroid adenocarcinoma	Resistant	Mutant (Arg175His)
4	TOV3041G	High grade serous	Sensitive	Nonsense
5	TOV3133G	High grade serous	Sensitive	Nonsense
6	TOV3133R	High grade serous	Resistant	Mutant (Gln192Ter)
7	OV90	High grade serous	Resistant	Mutant (Ser215Arg)
8	TOV3291G	High grade serous	Resistant	Mutant (Arg249Trp)
9	TOV21G	Clear Cells	Sensitive	Wild type
10	433 OVCA	High grade serous	Resistant	
11	HEY	Ovarian serous cyst adenocarcinoma	Resistant	Wild type
12	PA-1	Endometroid adenocarcinoma	Sensitive	Wild type

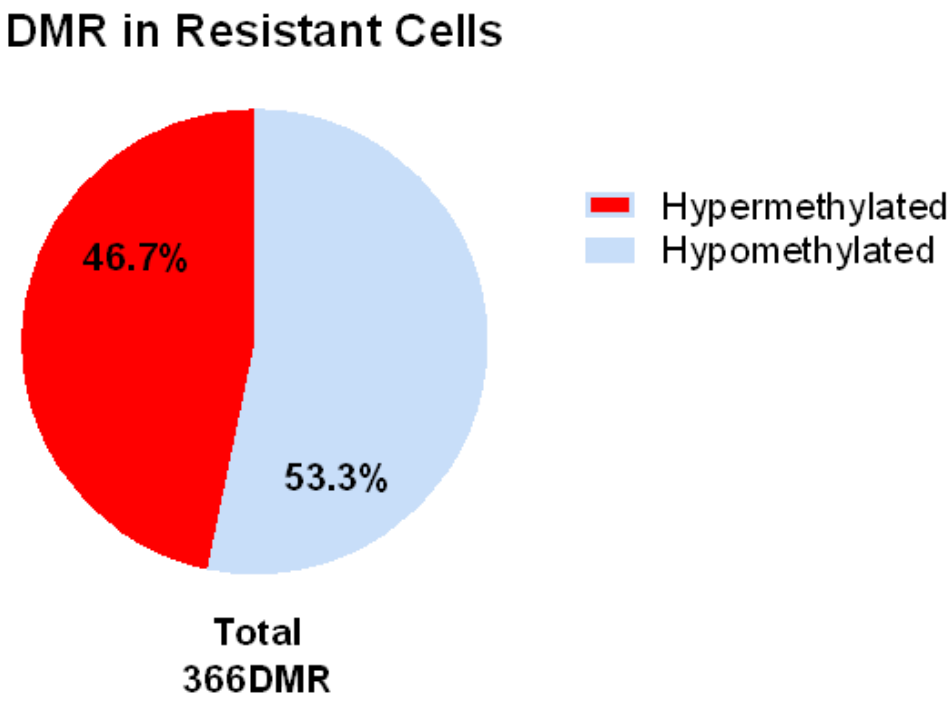

Figure 11. Global hypomethylation observed in resistant OVCA cells compared to sensitive cells. Global DNA methylation analysis of chemosensitive and chemoresistant OVCA cells of different histological subtypes was performed with Infinium HumanMethylation450 BeadChip assay.

v. Methylation changes induced by DNMT/TETs inhibitor alters the pGSN expression and CDDP-induced apoptosis in OVCA cells.

As previously discussed in the introductory section, epigenetic regulation of gelsolin expression by DNA methylation and histone modification has been identified in different cancer types[122,124,127,171,172], however, pGSN methylation in OVCA and its association with clinical outcomes has not been reported to date. We anticipate that hypomethylation in pGSN promoter region is associated with higher pGSN expression in chemoresistant OVCA cells compared to their chemosensitive counterpart. To validate this hypothesis, we induced hypermethylation and hypomethylation in chemoresistant and chemosensitive cells, respectively, by pharmacological inhibition of DNMTs and TETs and investigated its effect on pGSN expression and chemoresponsiveness of OVCA cells.

Chemoresistant (A2780cp) and sensitive (A2780s) OVCA cells were treated with BobCat339 – an inhibitor of TETs (TETi, 0-100 μM; 48 h) and 5-Azacytidine - an inhibitor of DNMTs (DNMTi, 0-10 μM; 48 h) respectively and harvested to quantify methylation ratio in pGSN promoter region (including ~600bp upstream region) by EpiTYPER technology. A2780cp and A2780s cells were also treated with CDDP (10 μM; 24 h) following TETi and DNMTi treatment and pGSN protein content as well as chemo-responsiveness (apoptosis %) were analyzed.

In our previous findings, we observed CDDP treatment was more effective in reducing pGSN expression and inducing apoptosis in chemosensitive cells compared to chemoresistant cells **(Fig. 7 & 8)**. Whereas TETi treatment alone appeared to have no significant influence on pGSN content and apoptosis in resistant cells, it markedly enhanced the CDDP-induced pGSN suppression and CDDP-induced apoptosis **(Fig. 12)**. In the absence of DNMTi treatment, CDDP decreased pGSN content and induced apoptosis. DNMTi treatment alone appeared to increase pGSN content in sensitive cells and markedly attenuated the CDDP-induced pGSN suppression; responses that was associated with suppressed CDDP-induced apoptosis **(Fig. 13)**. In endometrioid OVCA cells, methylation analysis of pGSN promoter region including 5`-upstream region revealed hypomethylation at five and hypermethylation at two CpG dinucleotides in resistant cells (A2780cp) compared to sensitive cells (A2780s) **(Fig. 14A) (Table 3).** Whereas DNMTi treatment in sensitive cells did not induce any change in pGSN methylation level, TETi

treatment in resistant cells appears to induce hypermethylation at five and hypomethylation at three CpG dinucleotides in pGSN promoter upstream region **(Fig. 14B) (Table 3)** suggesting hypomethylation role of TETs in the regulation of pGSN in OVCA cells. In the case of HGS (TOV3133R and TOV3133G), hypermethylation (average 68%) across all CpG sites at pGSN promoter region is observed in resistant cells (TOV3133R) and hypomethylation (average 8%) in sensitive cells (TOV3133G) **(Fig. 15A)**. Like our observation in endometrioid cells, DNMTi treatment did not induce any changes in pGSN methylation level in sensitive cells however TETi treatment appear to induce hypermethylation at one and hypomethylation at three CpG dinucleotides in pGSN promoter upstream region **(Fig. 15B) (Table 3)** suggesting methylation role of TETs in the regulation of pGSN in HGS cells as well.

Taken together, the above findings **(Fig. 12-16)** suggest that pGSN is regulated by DNA methylation, especially by TETs, in OVCA cells and is associated with chemo-responsiveness.

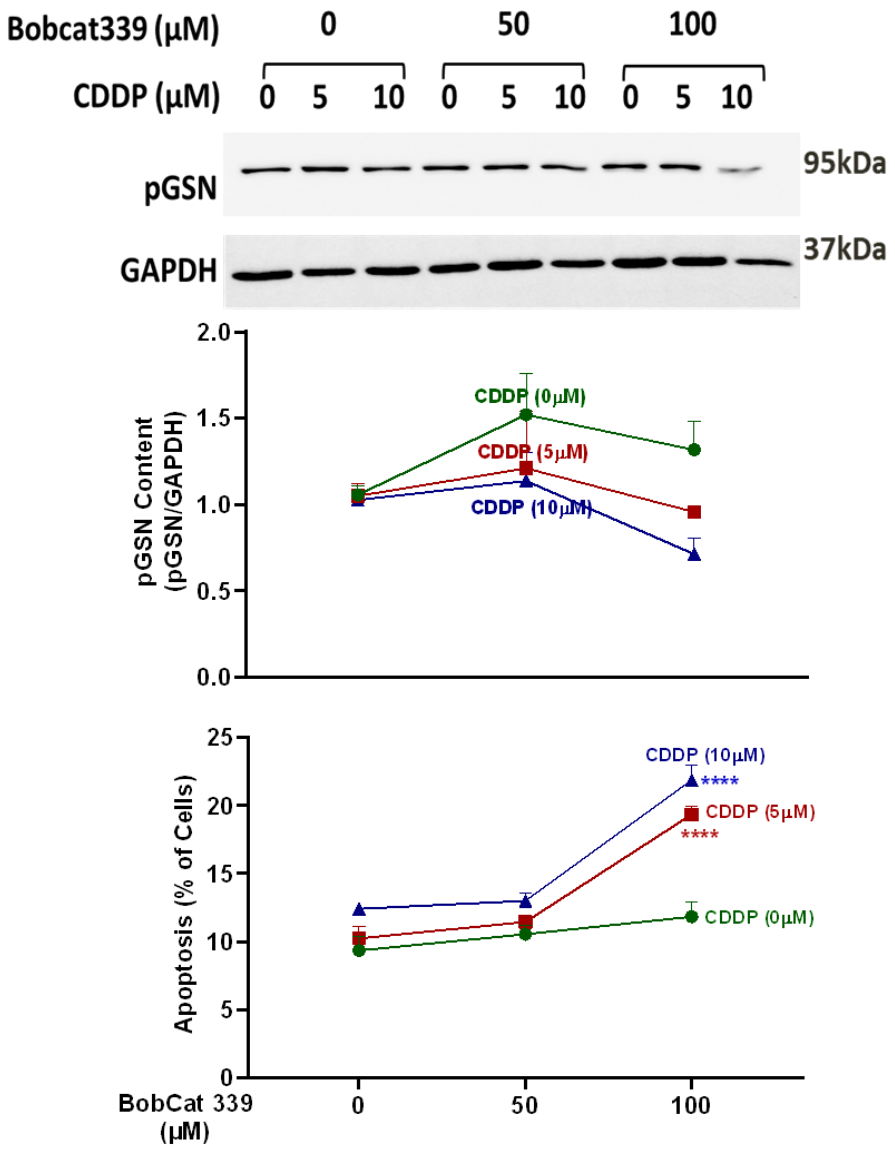

Figure 12. DNA methylation plays a role in the regulation of pGSN expression in OVCA cells. A2780cp cells were treated with BobCat 339; TETi (0-100 μM; 48 h) followed by CDDP treatment (10 μM; 24 h). pGSN and GAPDH (loading control) protein contents were measured by Western blotting and apoptosis was morphologically determined by Hoechst 33258 nuclear staining (mean ± SEM; n=3). *(p*<0.05, p**<0.01, p***<0.001, p****<0.0001 versus the corresponding control group: BobCat 339; 0 μM)*

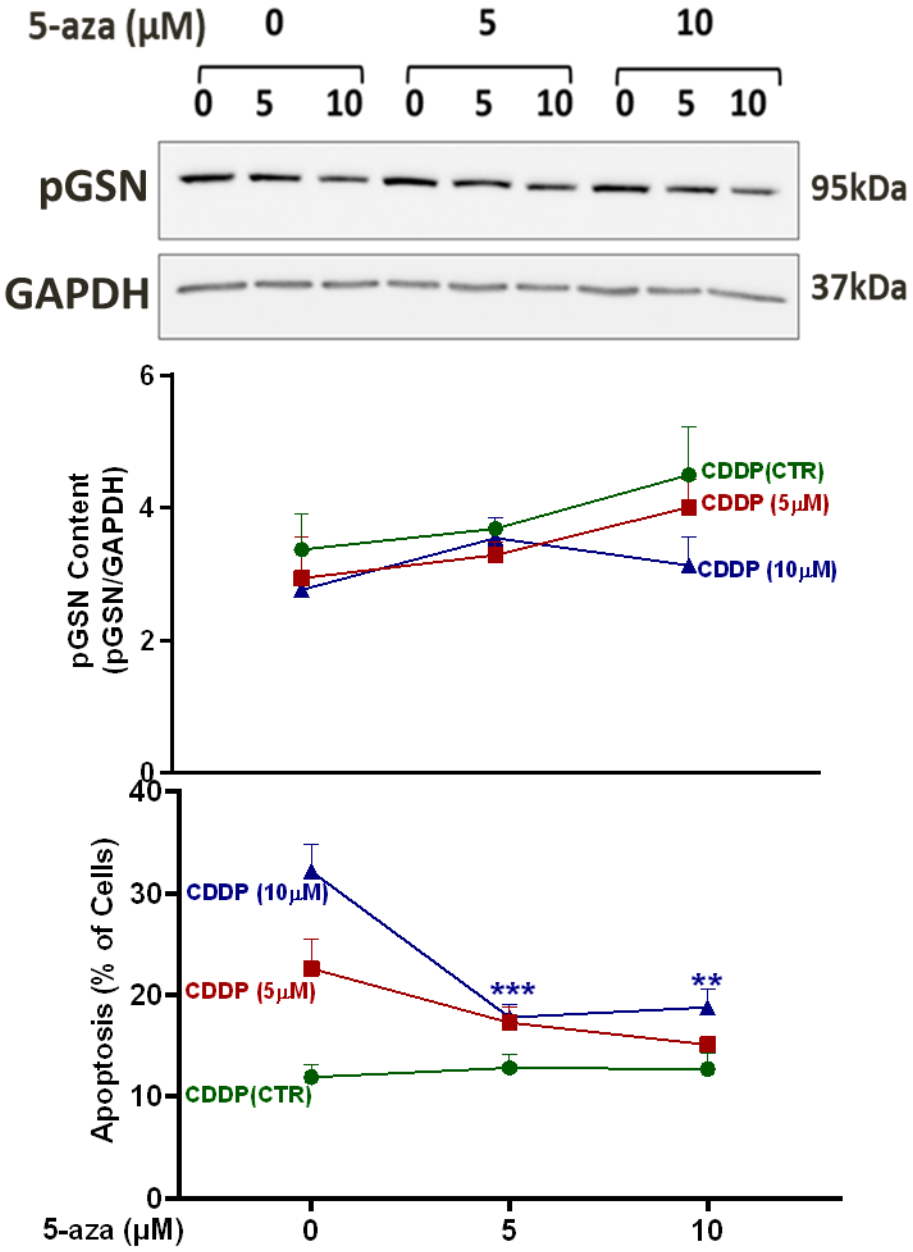

Figure 13. DNA methylation plays a role in the regulation of pGSN expression in OVCA cells. A2780s cells were treated with 5-aza; DNMTi (0-10 μM; 48 h) followed by CDDP treatment (10 μM;24 h) pGSN and GAPDH (loading control) protein contents were measured by Western blotting and apoptosis was morphologically determined by Hoechst 33258 nuclear staining (mean ± SEM; n=3). *(p*<0.05, p**<0.01, p***<0.001, p****<0.0001 versus the corresponding control group: 5-aza; 0 μM)*

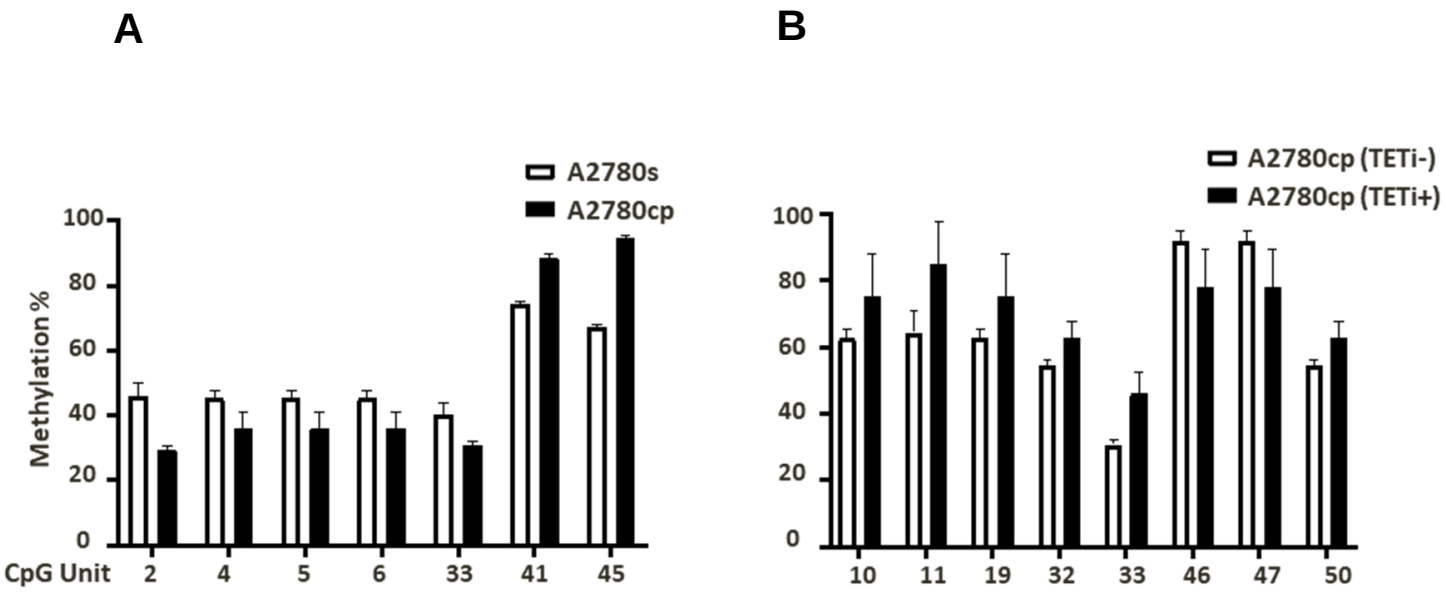

Figure 14. DNA methylation alter pGSN methylation in endometrioid OVCA cells. DNA methylation alters pGSN methylation in OVCA cells. Chemoresistant (A2780cp) and chemosensitive (A2780s) OVCA cells were treated with BobCat 339; TETi (100 μM; 48 h) and 5-aza; DNMTi (5 μM; 48 h), respectively. DNA methylation percentage was quantitatively assessed by EpiTYPER DNA methylation technology. Methylation % differences analyzed **(A)** at multiple CpG sites between endometrioid sensitive (A2780s) vs resistant (A2780cp) cells, and **(B)** at multiple CpG sites in endometrioid resistant cells after TETi treatment (mean ± SEM; n=3).

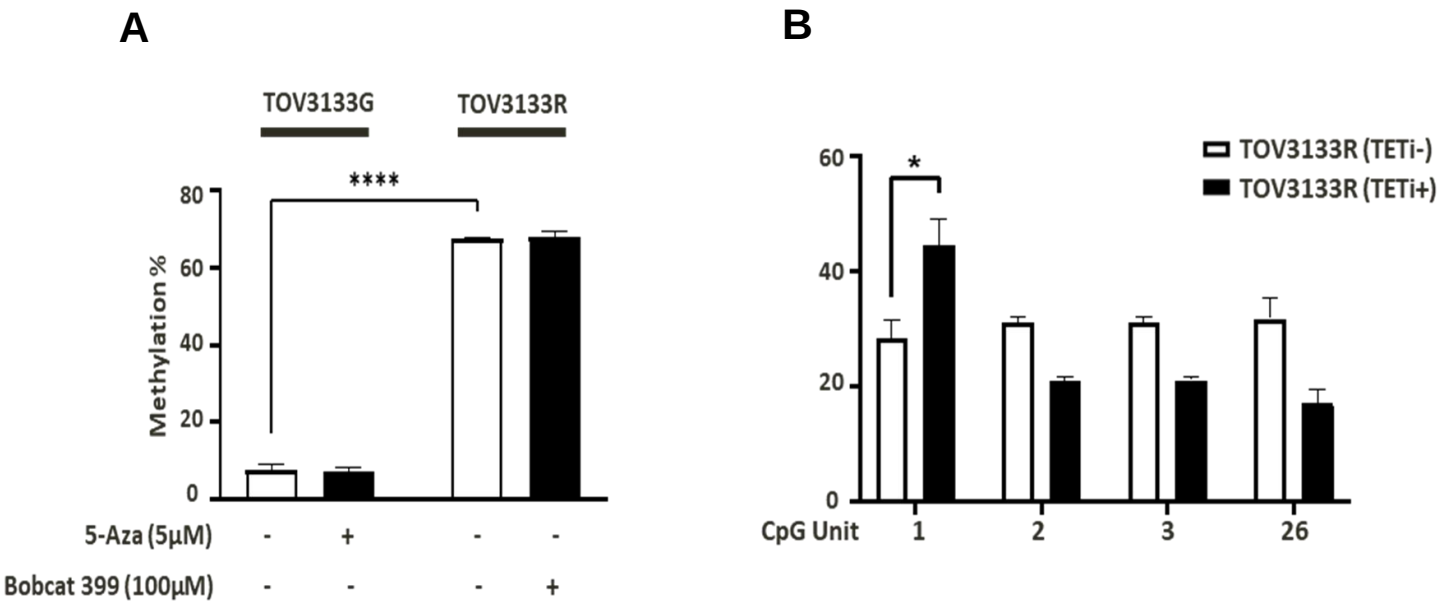

Figure 15. DNA methylation alter pGSN methylation in HGS OVCA cells. DNA methylation alters pGSN methylation in OVCA cells. Chemoresistant (TOV3133R) and chemosensitive (TOV3133G) OVCA cells of were treated with BobCat 339; TETi (100 μM; 48 h) and 5-aza; DNMTi (5 μM; 48 h), respectively. DNA methylation percentage was quantitatively assessed by EpiTYPER DNA methylation technology. Methylation % differences analyzed **(A)** between HGS sensitive (TOV3133G) and resistant (TOV3133R) cells, and **(B)** at multiple CpG sites in HGS resistant cells after TETi treatment (mean ± SEM; n=3).

Table 3 Methylation changes observed in multiple CpG sites at pGSN promoter region including 5`-upstream region (GRCH37-hg19: chr9:124061710-124062312).

CpG Unit	Position	CpG Unit	Position
CpG_1	chr9:124062312	**CpG_26**	chr9:124062052
CpG_2	chr9:124062303	**CpG_32**	chr9:124061980
CpG_3	chr9:124062293	**CpG_33**	chr9:124061970
CpG_4	chr9:124062281	**CpG_41**	chr9:124061872
CpG_5	chr9:124062279	**CpG_45**	chr9:124061833
CpG_6	chr9:124062274	**CpG_46**	chr9:124061826
CpG_10	chr9:124062211	**CpG_47**	chr9:124061824
CpG_11	chr9:124062189	**CpG_50**	chr9:124061710
CpG_19	chr9:124062127		

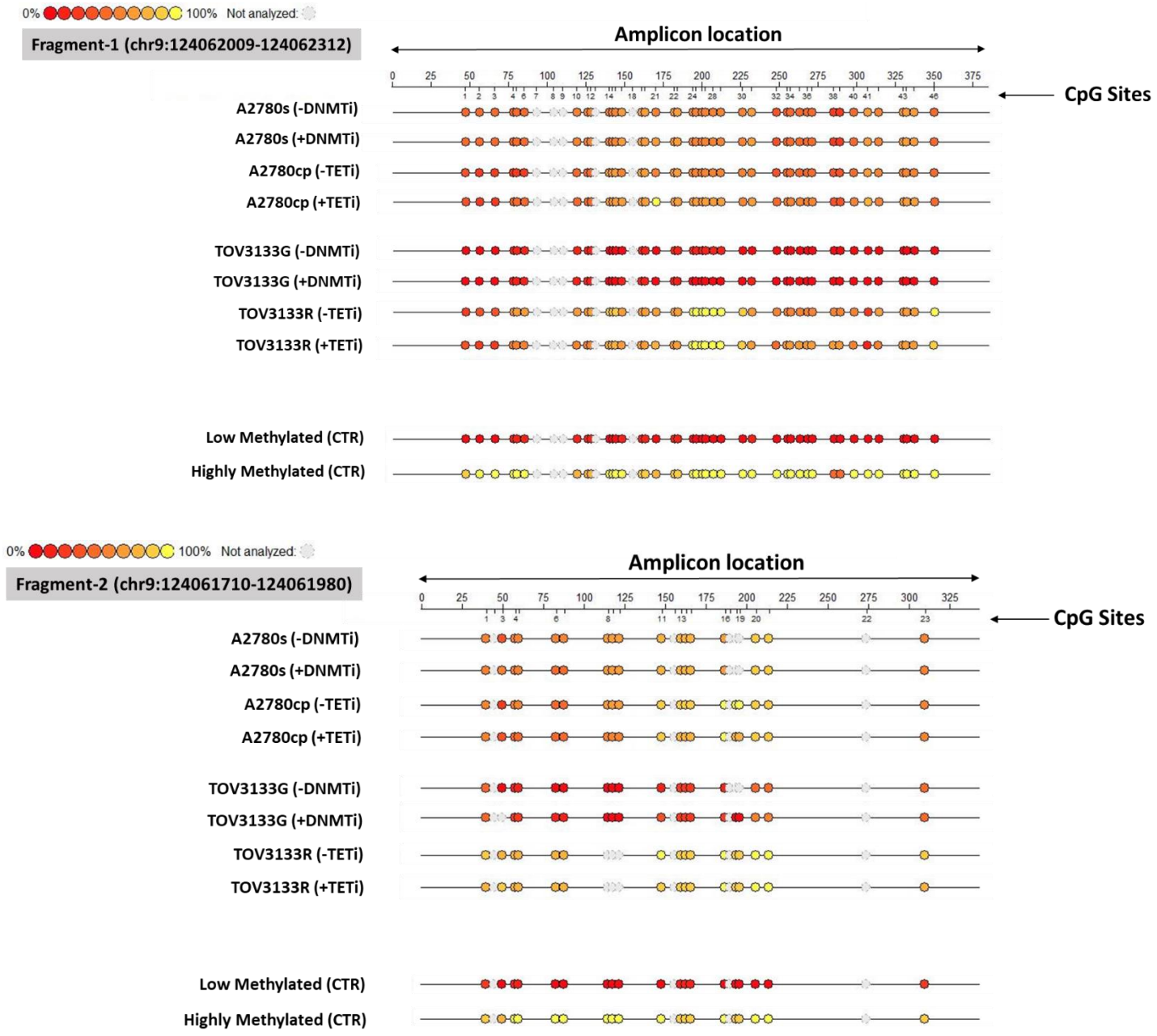

Figure 16. Schematic diagram of methylation status in OVCA cells. DNA methylation alters pGSN methylation in OVCA cells. Chemoresistant (A2780cp, TOV3133R) and chemosensitive (A2780s, TOV3133G) OVCA cells of were treated with BobCat 339; TETi (100 μM; 48 h) and 5-aza; DNMTi (5 μM; 48 h), respectively. DNA methylation percentage was quantitatively assessed by EpiTYPER DNA methylation technology. The region of interest for pGSN was analyzed in 2 fragments. Circles marks the position of CpG sites and color indicates the methylation status of CpG site. The color scale in each top left corner indicates the methylation level. Circle color from red to yellow indicates the methylation ranging from 0-100% (mean ± SEM; n=3).

vi. Knocking down of TET1 in chemoresistant cells increases the expression of pGSN and CDDP-induced cell death in OVCA chemoresistant cells.

Increasing evidence has revealed the role of TET1 in hypomethylating candidate genes that leads to cancer development and chemoresistance[151,155,156]. After confirming the role of DNA hypomethylation in the regulation of pGSN expression we further investigated specifically the role of TET1 in pGSN expression and pGSN-mediated chemoresistance in OVCA cells. We hypothesize that TET1 is involved in the upregulation of pGSN expression by demethylating its promoter region, resulting in chemoresistance in OVCA. To verify this, TET1 was overexpressed and silenced in chemosensitive and chemoresistant OVCA cells respectively and its effect on the pGSN content and chemo-responsiveness were analyzed.

Chemoresistant cells (A2780cp and TOV3133R) were transfected with three different siRNAs targeting TET1 (hs. Ri. TET1.13.1, hs. Ri. TET1.13.2, hs. Ri. TET1.13.3; 10nM) and negative scramble control siRNA (DS NC1; 10nM) with lipofectamine RNAiMax transfection reagent followed by CDDP treatment (10µM; 24 h). Transfection efficiency was confirmed with A2780cp cells using positive control (HPRT-S1 DS positive duplex control) and fluorescently labeled transfection control (TYE^{TM} 563 DS transfection control) **(Fig. 17)**. TET1 silencing was confirmed by assessing TET1 mRNA (qPCR). The effect of TET1 knockdown on pGSN mRNA abundance, protein content and CDDP-induced apoptosis were assessed using qPCR, Western blot and Hoechst staining, respectively. TET1 knockdown significantly increased pGSN protein content and CDDP-induced apoptosis in endometrioid resistant cells; A2780cp **(Fig. 18)**, whereas no significant TET1 knockdown observed in HGS resistant cells; TOV3133R **(Fig. 19).** This suggests that pGSN expression in endometrioid chemoresistant cells is inversely regulated by TET1 expression.

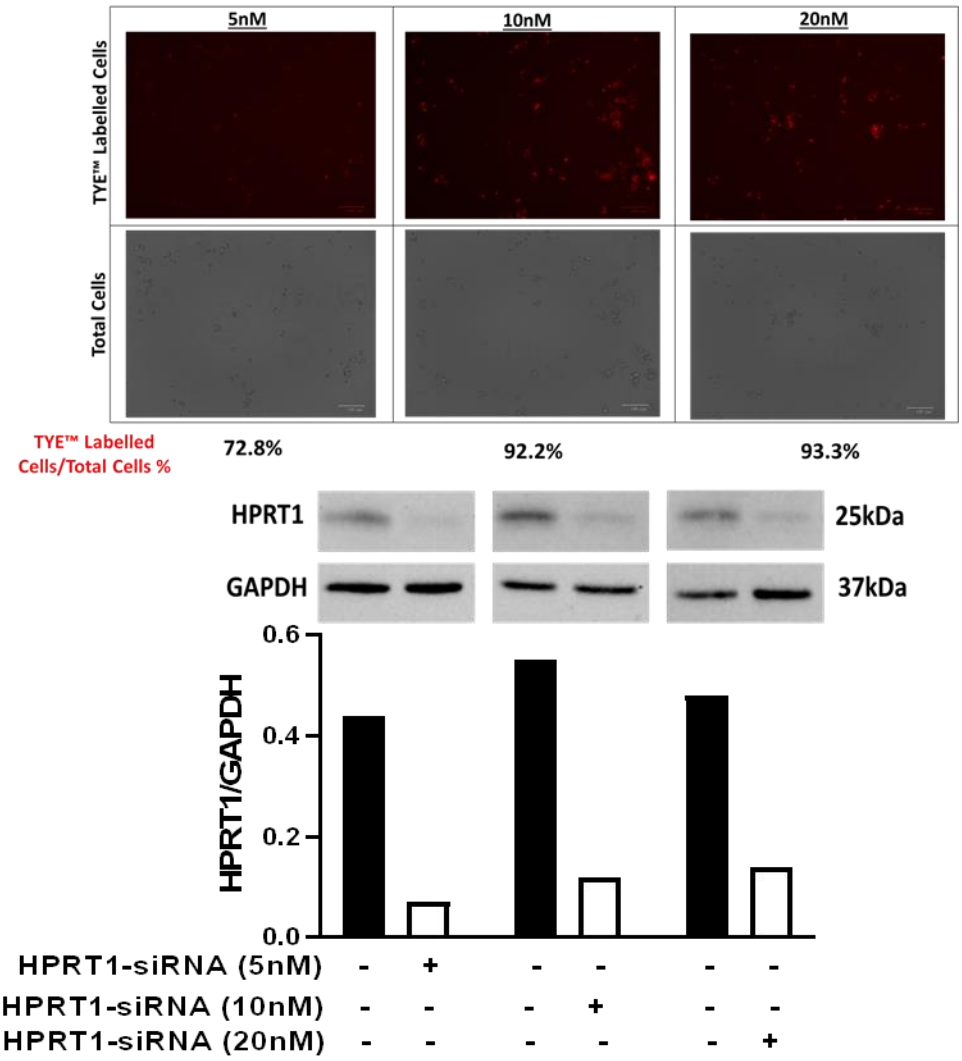

Figure 17. Confirmation of knockdown transfection efficiency. A2780cp cells were transfected with TYE™ 563 DS transfection control and HPRT-S1 DS positive duplex control (0-20 nM; 24 h). Transfection efficiency was morphologically observed under fluorescence microscope and further quantified by measuring HPRT1 protein content relative to GAPDH (loading control) by western blotting.

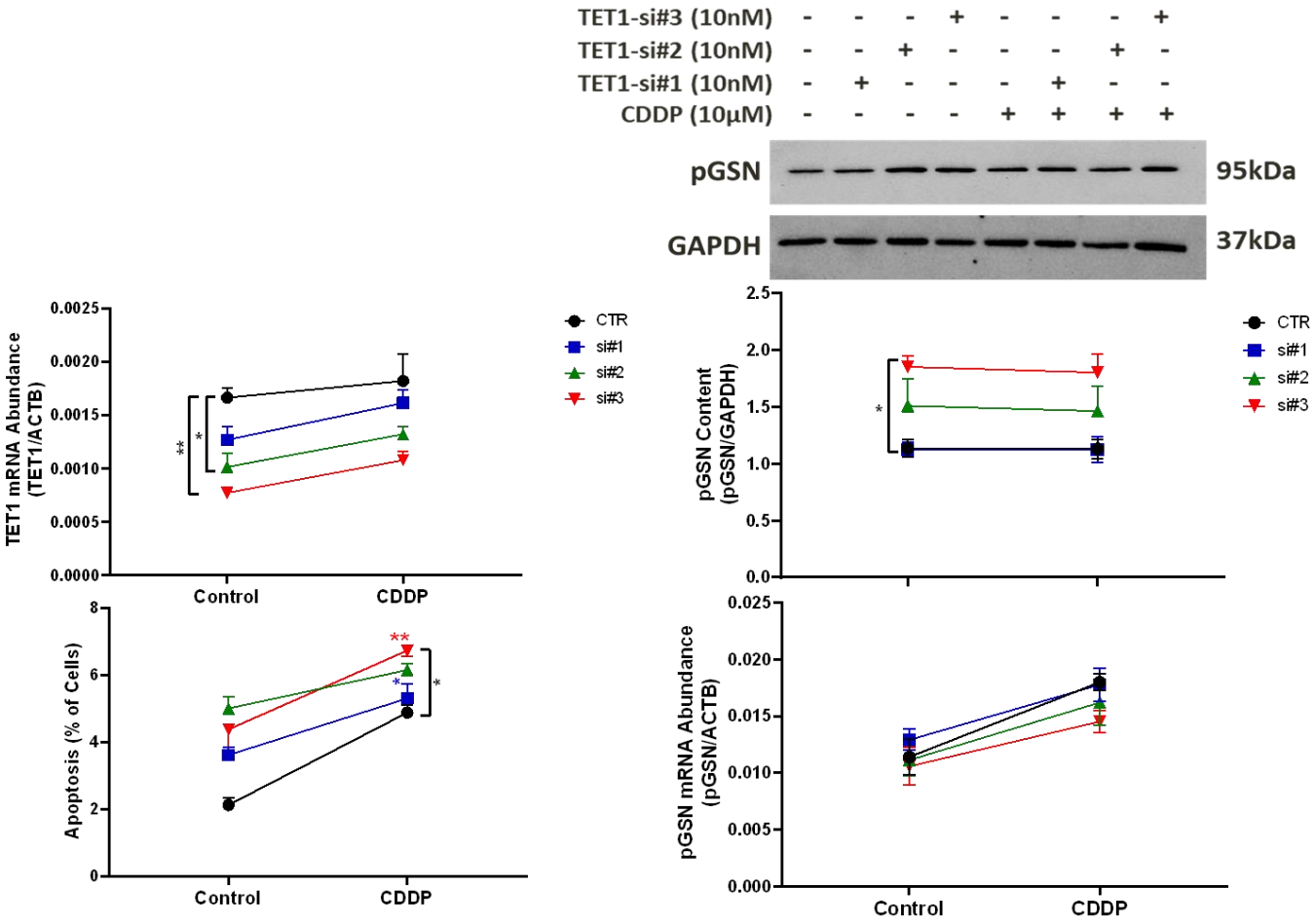

Figure 18. TET1 appears to have an inverse relationship with pGSN expression. A2780cp cells were transfected with three TET1-siRNAs (10 nM; 24 h) followed by CDDP treatment (10 μM; 24 h). mRNA contents relative to ACTB (loading control) were assessed by qPCR. pGSN and GAPDH (loading control) protein contents were measured by Western blotting and apoptosis morphologically determined by Hoechst 33258 nuclear staining (mean ± SEM; n=3). *p*<0.05, p**<0.01, p***<0.001, p****<0.0001*

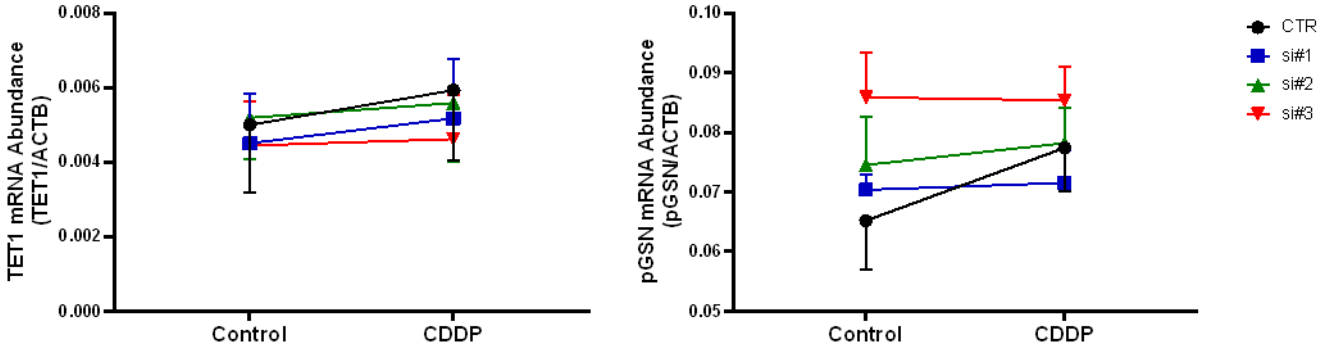

Figure 19. No significant TET1 knockdown observed in HGS resistant cells; TOV3133R. TOV3133R cells were transfected with three TET1-siRNAs (10 nM; 24 h) followed by CDDP treatment (10 µM; 24 h). mRNA contents relative to ACTB (loading control) were assessed by qPCR (mean ± SEM; n=3). *p*<0.05, p**<0.01, p***<0.001, p****<0.0001*

vii. TET1 overexpression in chemosensitive cells decreases the expression of pGSN and CDDP-induced cell death.

TET1 was overexpressed in chemosensitive cells (A2780s, TOV3133G) using TET1 cDNA (TET1 cDNA ORF clone, Human, SinoBiological; 0.5 µg) and an empty vector control (pCMV3-C-GFPSpark® vector, SinoBiological) with lipofectamine 2000 transfection reagent followed by CDDP treatment (10 µM; 24 h). TET1-cDNA concentration was optimized in A2780S using three different concentrations (0.5-2 µg; 24 h) **(Fig. 20)**.

TET1 expression was upregulated in chemosensitive cells (TET1-cDNA; 24 h) followed by CDDP treatment. TET1 over-expression was confirmed by assessing TET1 mRNA abundance (qPCR). The effect of TET1 overexpression on pGSN mRNA abundance, protein content and CDDP-induced apoptosis were assessed using qPCR, Western blot and Hoechst staining respectively. Overexpression of TET1 significantly reduced pGSN mRNA content in endometrioid sensitive cells (A2780s) but not pGSN protein content. Although TET1 overexpression significantly decreased CDDP induced apoptosis in A2780s cells **(Fig. 21)**, the pGSN mRNA abundance in HGS sensitive cells (TOV3133G) **(Fig. 22)** were not altered. Taken together loss-of-function and gain-of-function studies implied that pGSN expression in OVCA cells is inversely regulated by TET1 expression which is also associated with chemo-responsiveness.

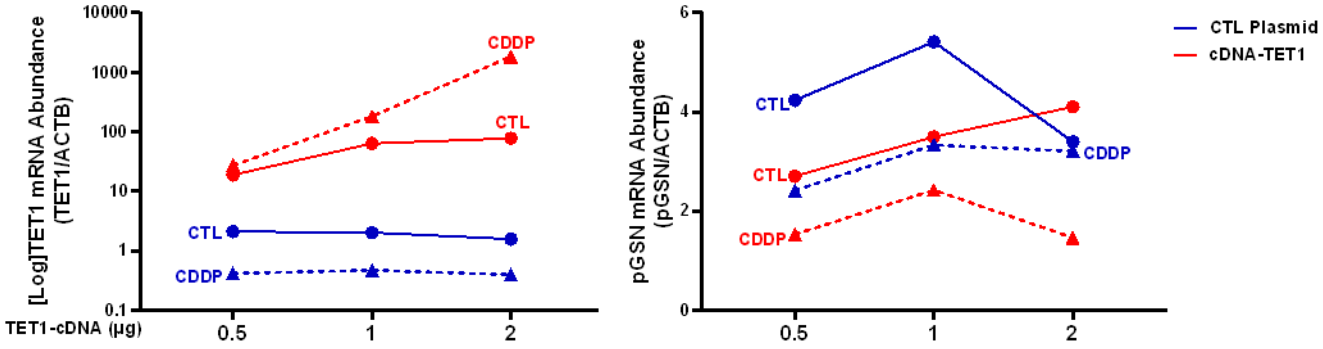

Figure 20. TET1-cDNA optimization. TET1-cDNA concentration was optimized in sensitive cells (A2780s) using three different concentrations (0.5-2 µg; 24 h). mRNA contents relative to ACTB (loading control) were assessed by qPCR.

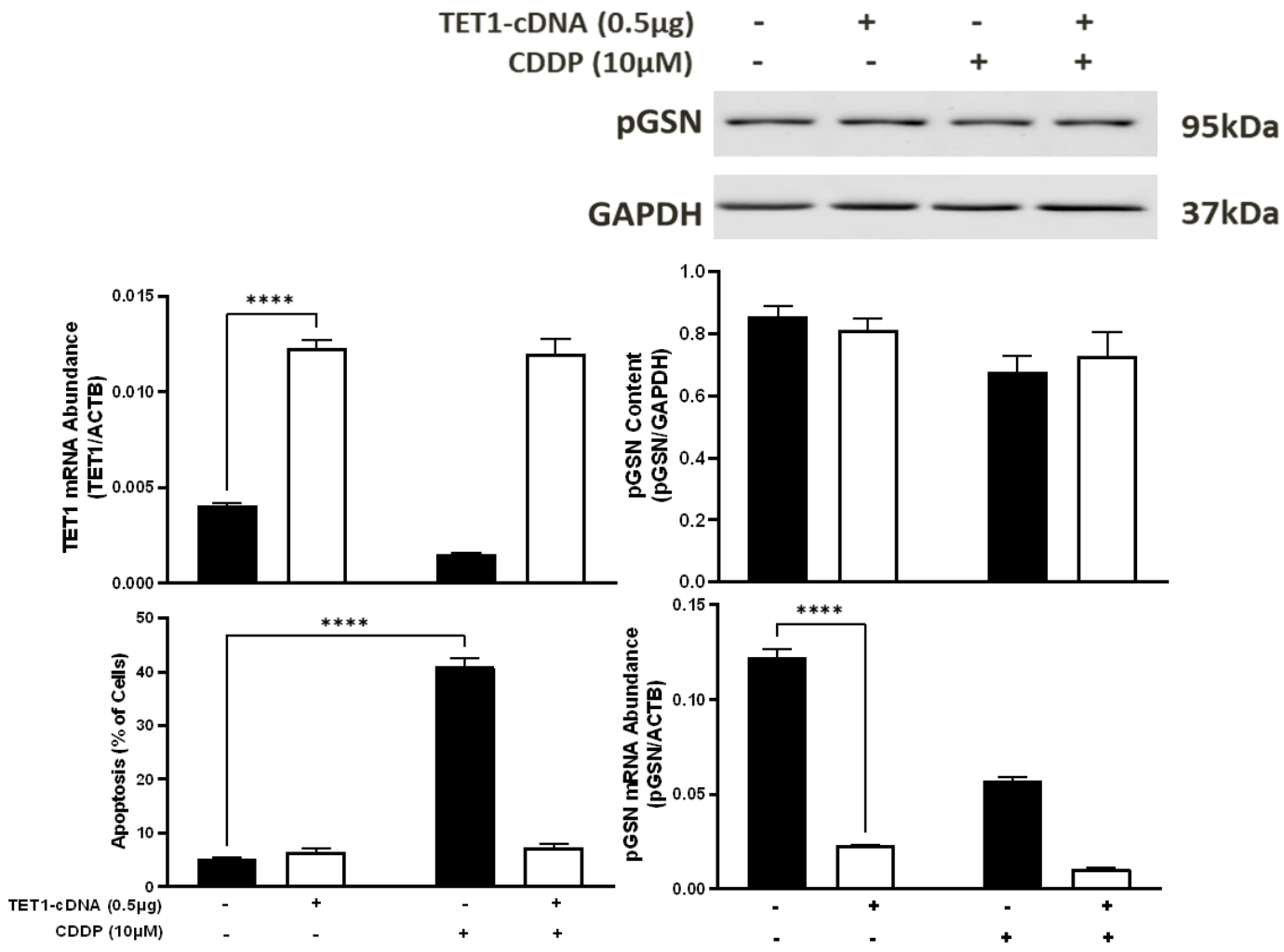

Figure 21. TET1 appears to have an inverse relationship with pGSN expression. A2780s cells were transfected with TET1-cDNA (0.5 µg; 24 h) followed by CDDP treatment (10 µM; 24 h). mRNA contents relative to ACTB (loading control) were assessed by qPCR. pGSN and GAPDH (loading control) protein contents were measured by Western blotting and apoptosis morphologically determined by Hoechst 33258 nuclear staining (mean ± SEM; n=3). *p*<0.05, p**<0.01, p***<0.001, p****<0.0001 compared to respective (-CDDP) group.*

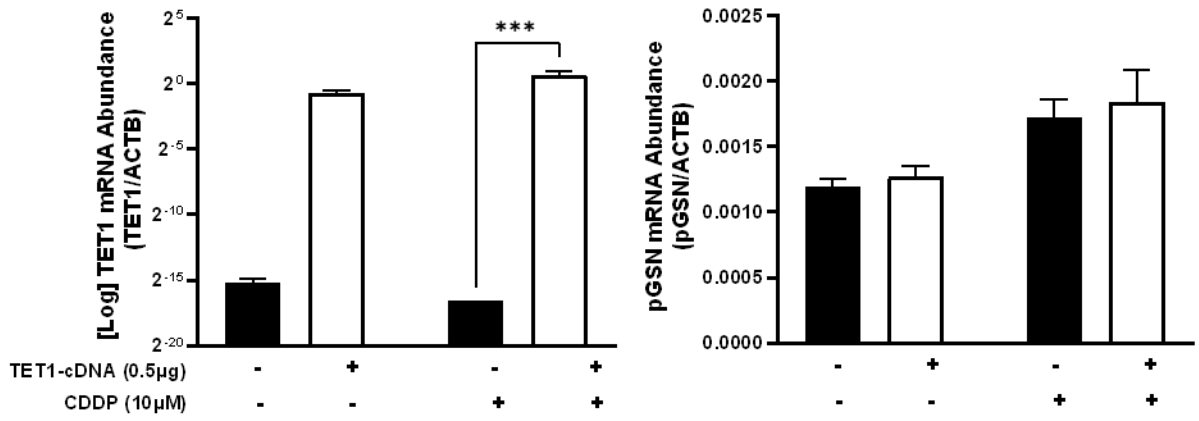

Figure 22. TET1 overexpression does not alter pGSN mRNA content in HGS sensitive cells. TOV3133G cells were transfected with TET1-cDNA (0.5 µg; 24 h) followed by CDDP treatment (10 µM; 24 h). mRNA contents relative to ACTB (loading control) were assessed by qPCR, (mean ± SEM; n=3). *p*<0.05, p**<0.01, p***<0.001, p****<0.0001 compared to respective (-CDDP) group.*

6. DISCUSSION

i. Chemoresistance Development: A Key Factor in Low Survival Rate of OVCA Patients

Ovarian cancer is the leading cause of gynecological cancer-related deaths among females[1]. Ovarian cancer patients have poor prognosis with overall low survival rate that ranges between 30-40%, which has not increased much since the last two decades[7]. Platinum-based chemotherapy with surgical debulking is the gold standard treatment approach for ovarian cancer[19]; however, its clinical effectiveness is greatly influenced by the chemoresistance development in ovarian cancer patients. The majority of ovarian cancer patients after initially responding to cisplatin, relapse and become resistant to cisplatin chemotherapy, causing over 90% of the deaths[45,46]. Thus, it is important to investigate molecular mechanisms underlying chemoresistance in OVCA patients for the development of molecular targeted strategies to overcome platinum resistance as well as improve overall survival rate of OVCA patients. Platinum resistance is a multifactorial phenomenon and many studies have proposed the involvement of multiple molecular pathways including mutation and silencing of tumor suppressor genes, activation of oncogenes, epigenetic modifications, dysregulation of apoptotic pathways, epithelial to mesenchymal transition, dysregulation in drug uptake and efflux, involvement of tumor microenvironment, enhanced DNA damage repair and the upregulation of gelsolin expression and function[49–54].

In this study, we identified that DNA methylation regulates pGSN expression and modify cisplatin sensitivity in OVCA cells. Gain-and-loss-of-function studies revealed inverse relationship between demethylating enzyme; TET1 and pGSN which was also associated with

chemoresponsiveness of OVCA cells. To best of our knowledge, this is the first study to report the role of DNA methylation and TET1 in the regulation of pGSN expression in OVCA cells.

ii. Possible role of pGSN and TET1 in OVCA

pGSN plays an important role in OVCA chemoresistance as well as other malignancies[56,112–115]. We detected high pGSN expression in OVCA chemoresistant cells compared to chemosensitive cells and its high expression was associated with decreased cisplatin responsiveness in OVCA cells. Also, significantly higher pGSN expression was associated with poor prognosis of OVCA patients[56]. Although, diagnostic and therapeutic importance of pGSN have been explored, we have yet to determine the underlying mechanisms for the differential pGSN expression between OVCA chemosensitive and resistant cells and how can it be regulated to improve chemoresponsiveness of OVCA patients.

In this study, we investigated if and how DNA methylation, specifically TET1-induced hypomethylation, plays any role in the regulation of pGSN expression and pGSN-induced chemoresistance in OVCA cells. TET1 is the most studied isoform of the TETs family and primarily responsible for 5mC to 5hmC oxidation, the first step in the demethylation mechanism[90,152,153]. Also, several studies have highlighted the possible role of TET1 as a promising target to overcome chemoresistance in different cancers[151,156–159]. Previous studies have shown that cisplatin-resistant endometrioid OVCA cells have increased levels of TET1 compared to sensitive cells which was associated with reduced apoptosis[151]. Contrary to previous findings, we have identified a relatively lower TET1 expression in endometrioid resistant cells compared to their sensitive counterpart. Meanwhile, TET1 was overexpressed in HGS resistant cells compared to sensitive cells. These findings suggest that TET1 expression in OVCA is histological subtype- specific, supporting the concept for its importance in personalized

therapies. Further studies are however, needed to investigate the different TET1 expression patterns in OVCA cells.

iii. DNA methylation in OVCA Chemoresistance

DNA methylation, both at gene-specific and global level, is a widely studied epigenetic mechanism that regulates expression of candidate genes involved in cancer development, progression, recurrence and chemoresistance[88–91]. The indispensable role of DNA methylation in ovarian cancer chemoresistance has been widely explored[94–99]. Although pGSN is transcriptionally regulated in OVCA chemoresistance, we have yet to determine how methylation affects its function. In our study, we foremost investigated the global hypomethylation in resistant cells compared to the sensitive cells which suggested that chemoresponsiveness of OVCA cells is associated with decreased global methylation percentage. We further examined the role of DNA methylation specifically in pGSN regulation and pGSN-induced chemoresistance. Pharmacological inhibitors of DNA methylation and demethylation altered pGSN protein levels and chemoresponsiveness of the OVCA cells, suggesting that DNA methylation regulates pGSN expression and affects pGSN-mediated cisplatin resistance in OVCA cells. The upstream region (~600bp) of pGSN promoter region is highly enriched with G/C and contains almost 69 CpG sites. We observed hypomethylation at multiple CpG sites in the pGSN promoter upstream region in resistant cells compared to sensitive cells. Further, we observed increased methylation at multiple CpG sites in the pGSN promoter upstream region after inhibiting TETs, suggesting that DNA methylation, specifically TETs induced hypomethylation appears to play a role in pGSN regulation in OVCA cells. Although TETi treatment increased methylation level of pGSN in resistant cells, this increase was not significant which could be explained by non-specificity of inhibitor as it acts globally to

induce hypermethylation across the DNA methylome. DNMTi/TETi used in our study could also potentially affect the expression of other methylation-dependent factors that regulate chemosensitivity in OVCA cells. Recently, targeted DNA methylation editing technologies has been developed including CRISPR/deactivated Cas9(dCas9)-based editing system which enables site-specific methylation changes and provide better approach to study relationship between methylation changes and gene expression[173]. dCas9-based recruitment of DNMTi/TETi to pGSN could be an effective approach to specifically modify pGSN methylation level and observe epigenetic regulation of pGSN.

The regulation of pGSN in ovarian cancer is complex. pGSN upregulates its own expression via the α5β1 integrins / FAK / Akt / HIF-1α axis[56]. It has been shown that DNA methylation can influence HIF-1α stability and subsequently affect expression of its downstream gene targets[174]. Hence, it is possible that treatment of methylating/demethylating agents resulted in DNA methylation changes in HIF-1α which subsequently modified pGSN expression. Another explanation for alteration in pGSN expression after inducing methylation changes could be the methylation changes in pGSN associated miRNAs which might have resulted in changes in their expression and ultimately pGSN expression. A number of miRNAs have been identified that regulate gelsolin expression in different cancers including miR-654-5p/450b5p in glioblastoma[128], miR-200a in hepatocellular carcinoma cells[175], and miR-9 in osteosarcoma[176]. miRNAs expression are regulated by epigenetic modification including DNA methylation[94,177,178] and conversely, miRNAs also regulate the expression DNMTs and TETs[179,180]. We suspect that inhibition of DNMTs and TETs resulted in alteration in one of the miRNAs that modified pGSN expression in OVCA cells.

DNA methylation is negatively correlated with chromatin accessibility and transcription factor binding sites, and act as a transcriptional repressor[181]. We have identified multiple transcription factor binding sites **(Table 4)** within GSN promoter sequence region that have previously been reported to be regulated by DNA methylation and are associated with chemoresistance in OVCA, including Signal transducer and activator of transcription (STAT3)[182,183], Msh homeobox 1 (MSX-1)[97], cAMP response element-binding protein (CREB), and octamer-binding protein (OCT-1)[184]. Treatment of DNMTi/TETi in OVCA cells might have resulted in transcriptional re-pression or activation of pGSN through these transcription factors. Therefore, further studies are needed to confirm which upstream mechanism was altered as a result of induced methylation/demethylation that ultimately affected pGSN expression.

Table 4. Transcription Factor Binding Sites identified within the GSN Promoter sequence region (prediction and visualization using Match 1.0 weight-based matrix program from TRASNFAC 6.0 (http://gene-regulation.com/pub/programs.html#match)

Name	Sequence	Position (0-based)	Strand	Score	p-value	E-value
STAT3 (M00497)	GGGTTCCC	179	-	7.35	0.00045	0.188
Msx-1 (M00394)	CACTAAATG	70	-	8.71	5.0E-5	0.0208
CREB (M00177)	CCTGACATACGG	98	-	9.05	0.000575	0.237
Oct-1 (M00162)	AGGCCTGACATACG	99	-	8.73	0.000975	0.40
YY1 (M00059)	TGCGCCCATTTAGTGTG	64	+	9.47	0.00015	0.061

STAT3; Signal transducer and activator of transcription, MSX-1; Msh homeobox 1, CREB; cAMP response element-binding protein, OCT-1; octamer-binding protein, YY1; YY1 transcription factor

iv. Relationship between TET1 and pGSN in OVCA chemoresistance

In this study, we investigated the role of TET1-induced demethylation in the pGSN regulation and OVCA chemoresistance. Multiple studies have reported the involvement of TET1 in hypomethylating candidate genes that leads to cancer development and chemoresistance[151,155,156]. TET1 is primarily responsible for 5-mC oxidation to 5-hmC, whereas other TET isoforms compensate for TET1 loss[185,186]. Information from OVCA public datasets showed TET1 expression in OVCA tissue is significantly associated with GSN expression (Fig.

6). Gain- and loss-of-function assays showed TET1 is inversely related to pGSN and positively related to chemoresponsiveness of OVCA cells **(Fig. 18 & 21)**. In our study, TET1 knockdown resulted in increased pGSN expression and increased chemoresponsiveness. This is contrary to previous studies where pGSN overexpression was significantly associated with decreased chemoresponsiveness of OVCA cells[56]. Silencing TET1 increases cisplatin sensitivity by altering the expression of multiple genes in various pathological conditions including vimentin in OVCA[151], and o6-methylguanine-DNA methyltransferase (MGMT) in oral squamous cell carcinoma (OSCC)[187]. This opposite relation observed between pGSN expression and chemoresistance after silencing TET1 could therefore be explained by the effect of other TET1 targets that suppressed the resistant effects of pGSN and enhanced cisplatin sensitivity. Our findings suggests that TET1 knockdown enhanced cisplatin sensitivity and induced apoptosis in resistant OVCA cells, while forced TET1 expression promoted chemoresistance in sensitive cells, irrespective of pGSN expression levels. Although we observed a negative relation between TET1 expression and pGSN expression in OVCA cells, we did not examine the methylation regulation of TET1 on pGSN promoter region. Hence, the underlying mechanisms upregulating pGSN expression after TET1 knockdown needs to be investigated.

Although, a number of studies have reported the involvement of TET1 in the regulation of cancer progression and chemoresistance development, the mechanism through which TET1 regulates gene expression remains controversial[155,158,188–191]. TET1 exerts tumor suppressor function by hypomethylating tumor suppressor genes and reactivating their expression[158,190,191]. However, emerging evidence has revealed oncogenic role of TET1-dependent and independent of its dioxygenase activity, suggesting the existence of its non-catalytic function in regulating gene expression[155,188,189]. A dual role of gelsolin as tumor suppressor and oncogene has been

observed in different human cancers[192]. Therefore, further studies with TET1 knockdown and overexpression are needed to determine whether the dysregulation in pGSN expression is because of pGSN promoter demethylation or due to transcriptional activation/repression by non-catalytic function of TET1.

v. Limitations and Resolutions

Although the findings from this study are compelling, we also acknowledge a few limitations of the study. We were unsuccessful in getting TET1 protein bands at the expected position (~235kDa) with Western blotting despite trying multiple antibodies. Another method to measure TET1 protein content such as ELISA, flow cytometry or other immunoassays could be used as an alternative approach for future studies.

Enzymatic activity of TET1 was assessed by measuring hydroxylase activity using OVCA cell lysate which might contain all TET isoforms, if expressed. This limitation could be resolved with TET1 purification through immunoprecipitation before measuring its enzymatic activity. However, the antibody used must be isoform specific.

TET1 knockdown conditions were optimized using endometrioid cell lines and we were not able to get significant knockdown with HGS cells. Despite being one of the most used cell line models, A2780 cell lines are not similar in their histopathological origin with HGSOC. Considering TET1 expression appears to be dependent on histology of cell lines, knockdown condition needs to be optimized in every histologic subtype cell line.

We did not measure methylation of pGSN promoter after TET1 knockdown, which should be included in future studies when investigating underlying mechanisms of TET1 inverse association with pGSN expression.

Lastly, this study was conducted using OVCA cell lines only, in-vivo models including animal models and patient-derived tissue need to be included in future studies to validate the role of TET1 in OVCA chemoresistance and its association with pGSN.

7. CONCLUSION AND FUTURE PERSPECTIVES

In summary, we have demonstrated that pGSN and TET1 are differentially expressed between OVCA chemosensitive and chemoresistant cells and their expression is also associated with chemoresponsiveness of OVCA cells. Furthermore, we revealed that DNA methylation, specifically TETs induced hypomethylation appears to regulate pGSN expression in OVCA cells and TET1 has an inverse relationship with pGSN expression.

DNA methylation is reversible and thus inhibition of DNA methylation/de-methylation can be considered as a promising therapeutic approach for OVCA treatment. This study provides mechanistic insights into the epigenetic regulation of pGSN relevant to chemotherapy resistance in OVCA. This regulatory mechanism of pGSN reveals a molecular basis for personalized OVCA therapy, and targeting pGSN methylation holds a great promise in overcoming OVCA chemoresistance and to improve overall OVCA patient survival. Epigenetic drugs when used in combination with chemotherapeutic drugs has reported to be more effective than either treatment alone[193]. Combination of pGSN-specific epigenetic drugs with chemotherapy may have potential in overcoming drug resistance in OVCA.

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