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Association between glutathione S-transferases M1 expression and treatment outcome in germ cell tumor patients

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Cisplatin-based chemotherapy is the mainstay in the treatment of germ cell tumors (GCTs). Glutathione S-transferases (GSTs) are polymorphic enzymes that catalyze the glutathione conjugation of alkylating agents, platinum compounds, and free radicals formed by chemotherapy and are thus implicated in developing treatment resistance. This study aimed to assess the expression level of GST mu 1 (GSTM1) and its association with treatment outcomes in patients with GCT. This translational study included tumor specimens from 207 patients with newly diagnosed GCTs, as well as cisplatin-sensitive GCT cell line xenografts and their resistant variants for all histological variants of GCTs. GSTM1 expression was detected by reverse transcription-quantitative PCR and immunohistochemistry using monoclonal antibodies, scored by the multiplicative quickscore (QS) method. GSTM1 expression was correlated with patient/tumor characteristics and treatment outcomes. The highest GSTM1 expression was observed in seminoma, followed by choriocarcinoma, embryonal carcinoma, and yolk sac tumor, while the lowest was observed in teratoma ($p<0.0001$). There was no association between GSTM1 expression in tumor tissue and patient/tumor characteristics. The low GSTM1 expression was associated with significantly better relapse-free survival compared with high GSTM1 (HR=0.50, 95% CI 0.23–1.09, $p=0.03$) but not overall survival (HR=0.61, 95% CI 0.24–1.54, $p=0.22$). Multivariate analysis showed that the prognostic value of GSTM1 was independent of the International Germ Cell Cancer Collaborative Group (IGCCCG) score. These data revealed the prognostic value of GSTM1 in GCTs, with a high GSTM1 expression associated with worse outcomes, suggesting that GSTM1 could be responsible, in part, for treatment resistance in GCTs.

Key words: germ cell tumors; glutathione S-transferase M1; prognosis; xenograft; resistance

Testicular germ cell tumors (GCTs) are the most common tumors in young men aged 20–40 years and represent a model of curable malignancy [1, 2]. Over 80% of metastatic GCTs achieve long-term remission due to cisplatin-based chemotherapy. However, the probability of cure varies from 50–98% depending on the prognostic group [3, 4]. Despite the excellent treatment outcomes, even in metastatic disease, a small proportion of patients experience disease recurrence and succumb to the disease. Cisplatin-resistant/refractory disease is associated with extremely poor prognosis, therefore the identification of new biomarkers and potential therapeutic targets in this setting is warranted [5, 6].

Cisplatin is the mainstay of GCT treatment [1]. It interacts with DNA, forming DNA-DNA and/or DNA-protein cross-links leading to the activation of several signaling pathways that initiate cell death [7]. Several mechanisms are involved in cisplatin resistance in GCTs. These include modulation of DNA repair capacity, altered apoptotic cell death pathways, deregulation of the expression of several specific genes, epigenetic alterations, decreased drug penetration, cisplatin efflux from cancer cells and others [8]. One of the mechanisms of cisplatin resistance is due to the ability of glutathione (GSH) to bind and inactivate cisplatin. Numerous studies showed that enzymatic inactivation of cisplatin by

glutathione S-transferases S-transferases (GSTs) contributes significantly to drug resistance [9, 10].

GSTs are polymorphic enzymes that catalyze the glutathione conjugation of alkylating agents, platinum compounds and free radicals formed by chemotherapy and thus implicated in the development of treatment resistance [9, 11]. The GSTs belong to a superfamily of proteins found widely in nature, occurring in both eukaryotes and prokaryotes [11]. GSTs have multiple biological functions, including protecting cells against oxidative stress and/or harmful substances. In addition, they play a role in the production and modification of leukotrienes and prostaglandins. GSTs offer protection to DNA against oxidative damage, which is associated with an increase in DNA mutations. They attach GSH to a broad range of hydrophobic and electrophilic substances, such as carcinogens, drugs and byproducts of oxidative metabolism. This makes them less toxic and more susceptible to further modification for discharge from the cell [12]. In addition, GSTs are included in several other catalytic reactions [13].

GSTs have been implicated in conferring resistance to various anticancer drugs across a range of cancers, including ovarian, head and neck, lung cancer and lymphomas. GST overexpression can potentially enhance the detoxification process of anticancer drugs [14]. Furthermore, several studies have uncovered synergistic interactions between GSTs and efflux pumps. These studies have demonstrated that GS-conjugates, formed through the action of GSTs, are actively transported out of cells by efflux transporters. Notably, transporters such as multidrug resistance protein 1 and P-glycoprotein, which belong to the superfamily of ATP-binding cassette transporters, are involved in this process [15].

Another mechanism through which GSTs are related to treatment resistance is the modulation of signal transduction pathways implicated in cell survival and apoptosis, such as the mitogen-activated protein kinase family [13]. GST mu 1 (GSTM1) is a member of the GST superfamily and one of the major GSH conjugation enzymes studied extensively in cancer. The genes encoding the mu class of enzymes are organized in a gene cluster on chromosome 1p13.3 and are known to be highly polymorphic. The mu class of enzymes, also known as GSTM, belongs to a family of phase II detoxification enzymes. These enzymes play a crucial role in cellular defense by catalyzing the conjugation of the antioxidant GSH with a wide range of electrophilic compounds, including xenobiotics (foreign substances such as drugs, pollutants and carcinogens) and endogenous compounds formed during oxidative stress [16]. Genetic variations of *GSTM1* can potentially alter an individual's vulnerability to carcinogens and toxins, impacting the effectiveness and toxicity of specific medications. Null mutations in the mu gene class have been associated with an elevated risk of various cancers, possibly due to heightened sensitivity to environmental toxins and carcinogens. Furthermore, this gene generates multiple protein isoforms through various transcript variants [16]. The role of *GSTM1* in cancer can be influenced by multiple factors, such as genetic polymorphisms, tumor microenvironment and treatment.

Numerous investigations have suggested that the absence of GSTM1 could potentially play a role in the development of malignancies, encompassing colon, liver and breast cancers [17–22]. Furthermore, GSTM1 plays a significant role in tumor immunity in colon cancer and is involved in cisplatin-induced ototoxicity and chemotherapy-induced peripheral neuropathy [23–25]. It was previously shown that combinations of GST genotypes were associated with primary and post-chemotherapy tumor histology and prognostic group presentation in patients with GCTs [26].

In the present study, we hypothesized that increased GSTM1 level leads to a decreased effectivity of platinum-based chemotherapy due to GSH conjugation of platinum compounds and free radicals in GCTs and due to modulations of signal transduction pathways implicated in cell survival and apoptosis. The aim of the present study was to assess the protein level of GSTM1 and its association with patient/tumor characteristics and treatment outcomes in patients with GCTs.

Patients and methods

Patients. This translational study included 207 patients with newly diagnosed GCT treated with systemic therapy at National Cancer Institute and St. Elizabeth Cancer Institute in (Bratislava, Slovakia), for whom paraffin-embedded tumor tissue samples were available in tissue biobank. All patients were treated with first line cisplatin-based therapy and there was no primary cisplatin-refractory patient. These institutes are national comprehensive cancer centers and referral centers for GCTs in Slovakia. Therefore, patient population in the present study and distribution of stages and histology don't represent this distribution in the general GCT population in Slovakia.

Patients underwent treatment between January 1999 and December 2013. Patients with concurrent malignancy other than nonmelanoma skin cancer in the previous five years were excluded. Patient/tumor characteristics, delivered systemic therapy and treatment outcomes were collected from all patients. This study received approval from the Institutional Review Board, and a patient consent waiver was obtained.

Diagnosis and tissue samples. Out of the total, 200 patients were diagnosed with primary testicular GCTs, with the remaining cases comprising biopsies from retroperitoneal and mediastinal masses in five and two instances, respectively. The classification of GCTs was carried out in accordance with the criteria established by the World Health Organization [27]. The tissue microarray was constructed as previously described [28]. Briefly, based on the tumor histology, one or two representative tumor areas from each histological subtype were pinpointed on hematoxylin and eosin sections.

These sections were then matched to their respective donor wax blocks. Using the multipurpose sampling tool Harris Uni-Core (MilliporeSigma), 3 mm diameter cores of the tumors were extracted from the donor blocks and placed into the recipient master block. The recipient block was later cut into 5 µm sections, and these sections were transferred to coated slides.

Cell lines. A range of human GCT cell lines were used in the present study, namely NTERA-2 (embryonal carcinoma, ATCC® CRL-1973™), JAR (choriocarcinoma, ATCC® HTB-144), JEG-3 (choriocarcinoma, ATCC® HTB-36™), NOY-1 (yolk sac tumor, catalog number: ENG101, FA: Kerafast) and TCam-2 (seminoma, generously provided by Dr. Kitazawa from Ehime University Hospital, Shitsukawa, Japan). In addition to the original cell lines, cisplatin-resistant variants of these cell lines were also employed. The cisplatin-resistant isogenic cell line variants, referred to as CisR, were generated by continuously culturing the cells in progressively higher concentrations of cisplatin (Pfizer, Inc.) for 6 months, as previously described [29, 30].

Gene expression analysis. Cultured cells were collected by trypsinization, and total RNA was isolated by NucleoSpin™ RNA (Macherey-Nagel) and treated with NucleoSpin™ rDNase Set (Macherey-Nagel).

Excised xenografts were homogenised using innuSPEED Lysis Tube A (Analytik Jena AG) and a rotor-stator homogenizer (Precellys® 24 Touch, Bertin Technologies SAS Parc d'activités du Pas du Lac). Total RNA was isolated using an innuPREP RNA Mini Kit 2.0 (Analytik Jena AG) and treated with NucleoSpin™ rDnase, according to the manufacturer's instructions (Macherey-Nagel).

RNA was reverse-transcribed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.). RT-qPCR for the GSTM1 gene was performed using TaqMan® Gene Expression Assays (Applied Biosystems; Thermo Fisher Scientific, Inc.). Briefly, the total PCR reaction mixture (20.0 µl) comprised 1.0 µl cDNA (100 ng), 10.0 µl TaqMan™ Fast Advanced Master Mix (cat. no. 4444558; Applied Biosystems; Thermo Fisher Scientific, Inc.), 8.0 µl nuclease-free water and 1.0 µl of the *GSTM1* (cat. no. Hs01683722; Applied Biosystems; Thermo Fisher Scientific, Inc.) TaqMan Gene Expression Assay. RT-qPCR was conducted using the AriaMx Real-time PCR System (Agilent Technologies Deutschland GmbH). The thermocycling conditions encompassed an initial step at 50 °C for 2 min to optimize Uracil N-glycosylase (UNG) activity, followed by an initial denaturation at 95 °C for 2 min. Subsequently, a total of 40 cycles were executed, comprising a two-step amplification process. This involved denaturation at 95 °C for 3 sec, followed by annealing-extension at 60 °C for 30 sec.

The acquired data were subsequently subjected to analysis using Agilent Aria software version 1.5 (Agilent Technologies Deutschland GmbH). The $2^{-\Delta\Delta Ct}$ method was used as a relative quantification strategy for qRT-PCR data analysis [31]. This method calculated relative *GSTM1* expression levels for examined samples; the sample used for reference was parental NTera2 (EC) cells. Expression levels were normalized to those of β-actin (TaqMan® Gene Expression Assays; Hs99999903) gene expression, serving as the housekeeping reference gene. Expression levels were normalized to those of β-actin (TaqMan® Gene Expression Assays; Hs99999903) gene expression, serving as the internal reference. The analysis cut-off value was set to Ct ≥35 (considered as the absence of target gene expression). All samples underwent analysis in technical triplicates, and the data are presented as the mean±SD. The statistical significance of fold changes in gene expression between groups was assessed using a Student's t-test.

Xenograft model. NOD scid gamma mice (age, 6–8 weeks; The Jackson Laboratory) were used in accordance with institutional guidelines under approved protocols, as previously described [32]. Briefly, a suspension of 2×10^6 of NTERA-2, NTERA-2 CisR, TCam-2, TCam-2 CisR, JEG-3, JEG-3 CisR, JAR, JAR CisR, NOY-1 and NOY-1 CisR cells in 100 µl of extracellular matrix (ECM) mixture 1:1 (50 µl serum-free DMEM or RPMI medium; 50 µl ECM) was prepared. Liquid bioreagent ECM Gel from Engelbreth-Holm-Swarm murine sarcoma (cat.no. E1270, Sigma-Aldrich) was used throughout the study. Mixture was subcutaneously injected into bilateral flanks of NOD scid gamma mice (total n=10). Xenograft dimensions were measured using a caliper, twice a week, and the animals were euthanized when one of the tumors reached a diameter of >10 mm. Euthanasia was performed by administration of isoflurane anesthesia 3.5% in a mixture with oxygen, and subsequent dislocation of the cervical vertebrae. Absence of a corneal reflex, failure to detect respiration and absence of a heart beat for a period of >5 min was used to confirm death. The tumors were examined by an experienced pathologist after excision, one excised tumor was snap-frozen in liquid nitrogen and stored at –80 °C until further processing, and the other was fixed in buffered formalin and paraffin-embeded for immunohistochemistry (IHC). One representative xenograft per each cell line model was taken for each analysis.

The project took place at the Animal Facility for Immunodeficient Mice of the Bio-medical Research Center SAS Bratislava, operating under license no. SK UCH 02017. This initiative obtained approval from both the Institutional Ethics Committee of the Bio-medical Research Center SAS Bratislava and the State Veterinary and Food Administration of the Slovak Republic, the latter of which serves as the national competence authority. The project was registered under no. Ro 1030/18-221 and was conducted in accordance with Directive 2010/63/EU and Regulation 377/2012, which aim to ensure the protection of animals used for scientific purposes.

Whole exome sequencing (WES). DNA from GCT parental and resistant cell lines was isolated with Quick DNA/RNA Miniprep Plus kit (Zymo Research Corp.), following the manufacturer's instructions. WES was

conducted in CeGaT, a provider of genetic diagnostics and next-generation sequencing services. The process included library preparation, sequencing and variant calling. Exome enrichment used the Twist Exome 2.0 kit (Twist Bioscience), with sequencing performed via 2×100 bp paired-end reads on an Illumina NovaSeq 6000 system. Alignment to the human reference genome (hg38) and variant calling adhered to the BWA-GATK best practices. CeGaT service also covers variant detection and their default and basic annotation according to the service standards (documentation of the variant calling and annotation is available on request). The Qiagen Clinical Insight platform (Qiagen AB) was employed for annotation, filtering and prioritization of variants, using a default somatic pipeline tailored for whole exome/whole genome sequencing that incorporates diagnostic and tumor site-specific information (e.g., JEG3-choriocarcinoma and placenta). Variant filtering was based on confidence scores, frequency and predicted functional impact. Pathogenicity criteria were adapted from The American College of Medical Genetics and Genomics (ACMG), Association for Molecular Pathology, American Society of Clinical Oncology and College of American Pathologists Guidelines [33, 34].

Immunohistochemistry. Slides were deparaffinized, rehydrated and immersed in phosphate-buffered saline solution (10 mM; pH 7.2). Tissue epitopes were demasked through heat-mediated antigen retrieval with citrate buffer (pH 6) at 98 °C for 20 min in Dako PT Link (Dako; Agilent Technologies, Inc.). Following tissue preparation on slides, the slides were incubated at room temperature for 60 min with a primary rabbit polyclonal anti-GSTM1 antibody (cat. no.ab244483; Abcam) that was diluted 1:100 in Dako REAL antibody diluent (Dako; Agilent Technologies, Inc.). Immunostaining was carried out using an anti-mouse/anti-rabbit immuno-peroxidase polymer (EnVision FLEX/HRP; Dako; Agilent Technologies, Inc.) for 30 min at room temperature, according to the manufacturer's instructions. To visualize the reaction, a diaminobenzidine substrate-chromogen solution (DAB; Dako; Agilent Technologies, Inc.) was applied for 5 min. Finally, the slides were counterstained with hematoxylin and eosin and mounted for analysis. GSTM1 positivity of epithelial cells in tubules in the kidney was used as a positive control, and the same tissue with the omition of the primary antibody served as a negative control.

Immunohistochemical stain scoring. Tumor cores and xenograft model tumors were independently evaluated by two observers (SH and ZC) blinded to clinicopathological data. In the case of disagreement, the result was reached by consensus. GSTM1 level was scored by the multiplicative quickscore (QS) method, which accounts both for a percentage of positive cells and staining intensity as previously described (28). Based on the QS value, GSTM1 level was categorized as either low (QS=0–9) or high (QS=10–18). Briefly, that high score means that a minimum of 60% of cells was positive with intermediate intensity. In the case of mixed GCTs, the highest GSTM1 level was used in any histological subtype for scoring and statistical analysis, as previously described [28].

Statistical analysis. Due to the non-normal distribution of GSTM1 QS values, as indicated by the Shapiro-Wilk normality test, nonparametric tests were employed for further analysis. The Mann-Whitney U test was used to assess differences in the distribution of GSTM1 level between two groups of patients. For comparisons among more groups, the Kruskal-Wallis test was performed. In cases where GSTM1 level was categorized as "low" or "high" based on the specified criteria, Fisher's exact or χ^2 tests were used for analysis.

The median follow-up period was determined by calculating the median observation time for all patients, including those still alive at their last follow-up. Progression-free survival (PFS) was calculated from the date of orchiectomy or tumor biopsy to the date of disease progression, death or last

Table 1. Patient characteristics.

Variable	N	%
All patients	207	100.0
Histology		
seminoma	37	17.9
non-seminoma	170	82.1
Tumor primary		
gonadal	200	96.6
retroperitoneal	5	2.4
mediastinal	2	1.0
Staging		
metastatic	169	81.6
non-metastatic	38	18.4
IGCCCG risk group		
good prognosis	117	56.5
intermediate prognosis	23	11.1
poor prognosis	29	14.0
Sites of metastases		
retroperitoneum	147	71.0
mediastinum	18	8.7
lungs	49	23.7
liver	11	5.3
brain	2	1.0
visceral non-pulmonary metastases	14	6.8
No of metastatic sites		
0	52	25.1
1 to 2	128	61.8
≥3	27	13.0
Pretreatment level of tumor markers, median (range)		
AFP (mIU/ml)	7 (0–60.470)	
HCG (IU/ml)	4 (0–929.000)	
LDH (mkat/l)	6 (2–87)	

Abbreviations: AFP-alpha-fetoprotein; HCG-human chorionic gonadotropin; IGCCCG-International Germ Cell Collaborative Group; LDH-lactate dehydrogenase

follow-up. Overall survival (OS) was calculated from the date of orchiectomy or tumor biopsy to the date of death or last follow-up. The Kaplan-Meier product limit method was used to estimate PFS and OS, and a log-rank test was employed to compare survival between groups. To assess the impact of GSTM1 level in the primary tumor and prognosis based on International Germ Cell Cancer Collaborative Group (IGCCCG) criteria, a multivariate Cox proportional hazards model was used for both PFS and OS. Statistical analysis was conducted using NCSS 2019 software (NCSS 2019 Statistical Software, NCSS, LLC). A p-value <0.05 was considered to indicate a statistically significant difference.

Results

GSTM1 level in GCTs. Patients' characteristics are summarized in Table 1 and Supplementary Table S1. The median age of patients was 30 years (16-67 years). The majority of patients had good risk, metastatic testicular cancer with non-seminoma histology. Tumor specimens from 207 patients before the administration of systemic therapy included 37 pure seminomas, 72 embryonal carcinomas (EC), 18 yolk sac tumors (YST), 4 choriocarcinoma (ChC) and 13 immature teratomas. Sixty-three tumors were mixed GCTs.

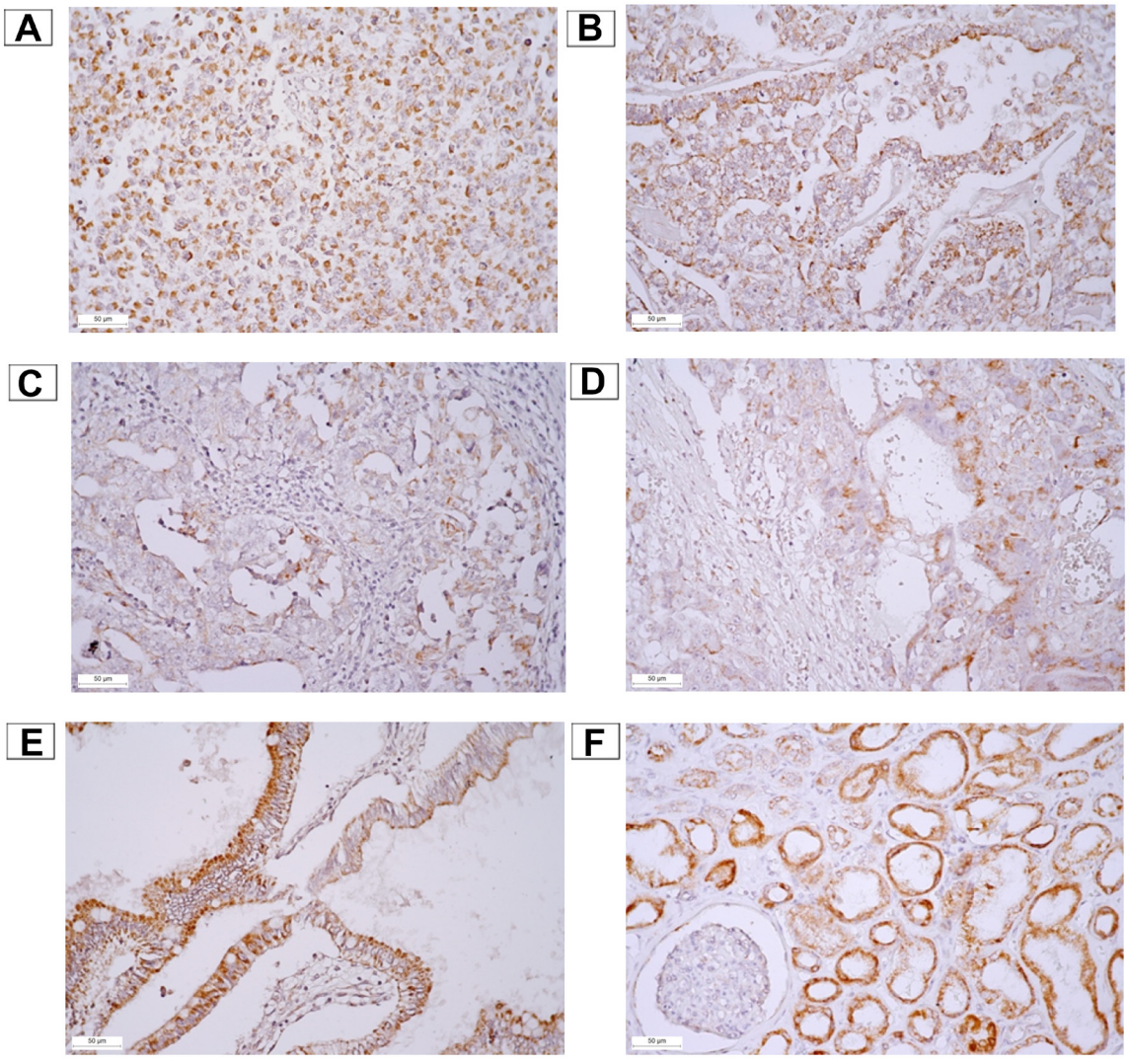

Figure 1. Immunohistochemical detection of GSTM1 expression in testicular tumors. A) Diffuse strong cytoplasmic positivity in seminoma. B) Diffused strong to moderate cytoplasmic positivity in yolk sac tumor. C) Focal moderate to weak cytoplasmic positivity in embryonal carcinoma. D) Focal strong and moderate cytoplasmic positivity in choriocarcinoma. E) Diffused strong to moderate cytoplasmic positivity in epithelial cells of teratoma. F) kidney tissue with positivity in the tubules and negativity in the glomeruli. Original magnification ×400.

The mean±standard deviation (SD) of GSTM1 level detected by IHC in GCTs was 5.48+7.76. The highest level was observed in seminoma (7.06±4.55), ChC (6.42±5.00), embryonal carcinoma (3.60±3.46) and YST (3.56±3.68), while the lowest was in teratoma (1.16±2.08) (p<0.0001; Figure 1; Supplementary Table S2).

There was no association between GSTM1 level in tumor tissue and patient/tumor characteristics (Table 2, Supplementary Tables S3, S4).

Prognostic value of GSTM1. In the median follow-up of 81.1 months (0.3–235.8 months), 44 (21.9%) patients experienced disease progression, and 31 (15.4%) patients

Table 2. Association between GSTM1 and Patient/tumor characteristics.

Variable	Number of patients	Mean[a] QS	Median	SD	SEM	p-value	Low QS (QS<10)		High QS (QS≥10)		p-value
							N	%	N	%	
Histology											
seminoma	37	5.89	6.00	4.07	1.28	0.11	28	75.7	9	24.3	0.36
non-seminoma	170	5.39	4.00	8.35	0.60		140	82.4	30	17.6	
Tumor primary											
gonadal	200	5.51	4.00	7.87	0.55	0.49	162	81.0	38	19.0	1.00
extragonadal	7	4.57	4.00	2.82	2.94		6	85.7	1	14.3	
Staging											
metastatic	169	5.67	5.00	4.02	1.25	0.58	137	81.1	32	18.9	0.82
non-metastatic	38	4.61	4.00	3.89	1.26		32	84.2	6	15.8	
IGCCCG risk group											
good	117	5.94	5.00	9.67	0.72	0.20	94	80.3	23	19.7	0.92
intermediate	23	6.00	5.00	4.23	1.62		18	78.3	5	21.7	
poor	29	4.34	3.00	4.12	1.44		24	82.8	5	17.2	
Sites of metastases											
retroperitoneum											
no	60	4.62	4.00	3.78	1.00	0.20	50	83.3	10	16.7	0.70
yes	147	5.83	5.00	8.87	0.64		118	80.3	29	19.7	
mediastinum											
no	189	5.59	4.00	8.00	0.56	0.26	154	81.5	35	18.5	0.75
yes	18	4.33	2.50	4.35	1.83		14	77.8	4	22.2	
lungs											
no	158	5.72	4.00	8.57	0.62	0.48	126	79.7	32	20.3	0.41
yes	49	4.71	4.00	4.14	1.11		42	85.7	7	14.3	
liver											
no	196	5.56	4.00	7.89	0.55	0.19	159	81.1	37	18.9	1.00
yes	11	4.00	2.00	4.69	2.34		9	81.8	2	18.2	
brain											
no	205	5.45	4.00	7.78	0.54	0.20	167	81.5	38	18.5	0.34
yes	2	8.00	8.00	2.83	5.49		1	50.0	1	50.0	
visceral non-pulmonary metastases											
no	193	5.54	4.00	7.95	0.56	0.59	156	80.8	37	19.2	1.00
yes	14	4.57	3.50	4.15	2.08		12	85.7	2	14.3	
No of metastatic sites											
0	52	4.52	4.00	3.84	1.08	0.30	44	84.6	8	15.4	0.74
1 to 2	128	6.05	5.00	9.32	0.69		102	79.7	26	20.3	
≥ 3	27	4.63	3.00	4.32	1.49		22	81.5	5	18.5	
S stage											
S0	76	6.24	4.00	11.58	0.90	0.95	62	81.6	14	18.4	0.99
S1	81	5.06	4.00	4.08	0.87		66	81.5	15	18.5	
S2	29	4.86	4.00	4.40	1.45		24	82.8	5	17.2	
S3	19	4.79	4.00	4.25	1.79		15	78.9	4	21.1	

Abbreviations: Gstm1-glutathione S-transferases M1; IGCCCG-International Germ Cell Collaborative Group; Note: [a]the highest GSTM1 expression in any histological subtype in case of mixed germ cell tumor

died. The estimated 5-year PFS and OS were 85.9% (95% CI 79.5–92.3%) and 90.2% (95% CI 84.7–95.7%), respectively. The low GSTM1 level (QS<10) was associated with a significantly better PFS compared with the high GSTM1 (QS≥10) (HR=0.50, 95% CI 0.23–1.09, p=0.03) but not OS (HR=0.61, 95% 0.24–1.54, p=0.22) (Figure 2). Subgroup analysis revealed the prognostic value of GSTM1 in patients with testicular primary tumors, IGCCCG good risk, retroperitoneal metastases, and without mediastinum, lung and brain involvement, and in patients with one to two metastatic sites for PFS, while in patients with IGCCCG good risk and visceral non-pulmonary metastases GSTM1 was associated with OS (Table 3). Patients with seminoma or liver metastases with a low GSTM1 QS score had a trend for better OS compared to patients with tumors with high QS.

Multivariate analysis showed that the prognostic value of GSTM1 was independent of the IGCCCG score (Table 4).

GSTM1 expression analysis in GCT cell lines and xenografts. Next, the GSTM1 expression was analyzed in established human GCT cell lines and their isogenic variants with acquired resistance to cisplatin. The GSTM1 expression was determined both in cultured cells and corresponding xenografts (Supplementary Table S5). The highest GSTM1 expression was detected in cells and xenografts from NTERA-2 (EC). A significant increase in GSTM1 expression was detected in the cisplatin-resistant NTERA-2 CisR cells (p≤0.05). Resistant TCam-2 CisR (SE) cells exhibited loss of GSTM1 expression and a significant decrease in xenografts (p≤0.01) compared with parental TCam-2 (SE). The same trend was identified in choriocarcinoma cell line pair JAR and JAR CisR cells: Significant decrease in the expression level in resistant cells and xenografts (p≤0.01 and p≤0.05, respectively). In JEG-3 choriocarcinoma cells GSTM1 expression was not detected; however, a low GSTM1 expression was detected in JEG-3 CisR cells and in xenografts derived from these cells. GSTM1 expression was not detected in NOY-1 (YST) and NOY-1-resistant cells or xenografts (Figure 3).

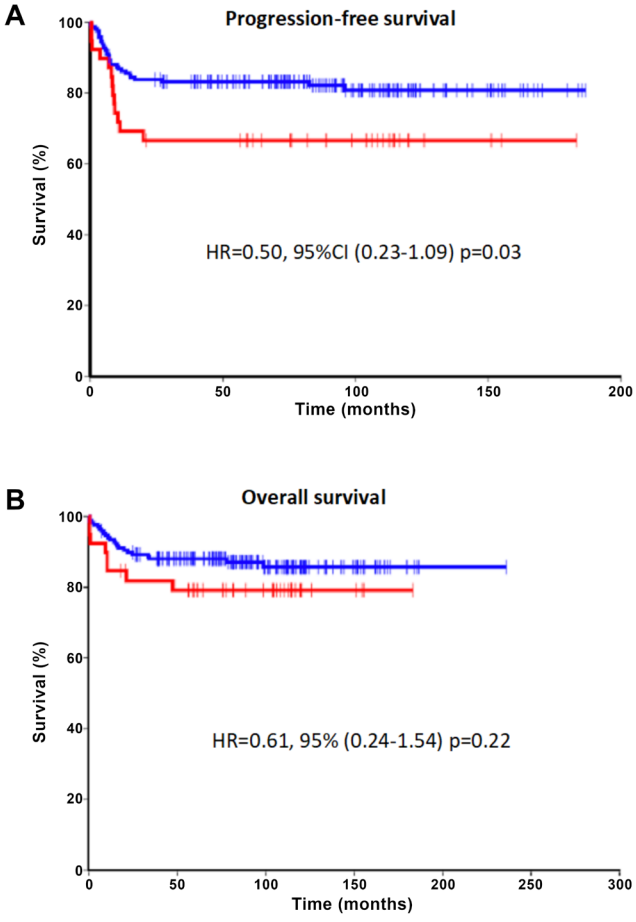

Figure 2. Kaplan-Meier estimates of probabilities of progression-free survival A) according to GSTM1 expression in germ cell tumor patients (n=207), HR=0.50, 95%CI 0.23–1.09, p=0.03; and overall survival B), HR=0.61, 95% 0.24–1.54, p=0.22; 1-high GSTM1, red line (QS≥10); 0-low GSTM1, blue line (QS<10).

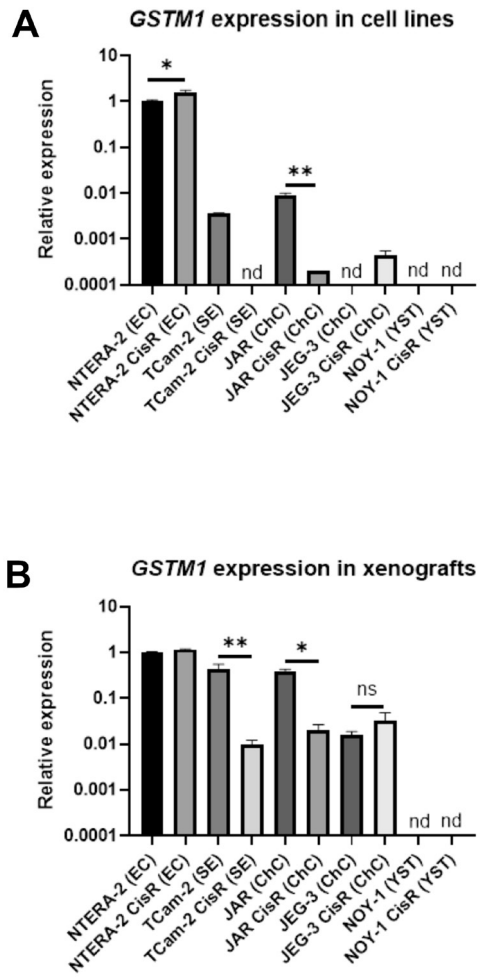

Figure 3. Relative expression of the GSTM1gene in GCT cells (A) and xenografts (B).

Table 3. Prognostic value of GSTM1.

Variable	Progression-free survival				Overall survival			
	HR	Lower 95% CI	Upper 95% CI	p-value[a]	HR	Lower 95% CI	Upper 95% CI	p-value[a]
All patients	0.5	0.23	1.09	0.03	0.61	0.24	1.54	0.22
Histology								
seminoma	0.28	0.04	1.93	0.09	0.14	0.01	2.17	0.06
non-seminoma	0.55	0.23	1.3	0.11	0.75	0.28	2.01	0.53
Tumor primary								
gonadal	0.48	0.22	1.06	0.03	0.58	0.23	1.5	0.19
extragonadal	0	0	0	0.68	0	0	0	0.66
IGCCCG risk group								
good	0.29	0.08	1.03	0.01	0.23	0.03	1.79	**0.048**
intermediate	0.41	0.07	2.34	0.20	0.73	0.13	4.3	0.71
poor	1.04	0.3	3.57	0.95	0.81	0.21	3.11	0.74
Sites of metastases								
retroperitoneum								
no	0.58	0.04	8.29	0.63	0.61	0.04	8.34	0.66
yes	0.5	0.22	1.13	0.04	0.63	0.23	1.68	0.29
mediastinum								
no	0.39	0.16	0.95	0.006	0.52	0.18	1.49	0.14
yes	2.61	0.55	12.4	0.35	1.55	0.24	10.18	0.69
lungs								
no	0.4	0.15	1.11	0.03	0.55	0.14	2.17	0.32
yes	0.5	0.13	2.02	0.21	0.4	0.09	1.83	0.09
liver								
no	0.52	0.23	1.2	0.07	0.68	0.24	1.9	0.41
yes	0.29	0.03	3.22	0.12	0.23	0.02	3.19	0.06
brain								
no	0.51	0.23	1.15	0.048	0.65	0.25	1.71	0.32
yes	NR				NR			
visceral non-pulmonary metastases								
no	0.51	0.22	1.18	0.06	0.65	0.23	1.86	0.37
yes	0.27	0.02	3.28	0.09	0.22	0.01	3.33	**0.04**
No of metastatic sites								
0	0	0	0	0.54	0	0	0	0.52
1 to 2	0.43	0.17	1.09	0.02	0.58	0.17	1.93	0.30
≥ 3	0.68	0.16	2.92	0.56	0.51	0.1	2.56	0.31
S stage								
S0	0.35	0.04	3.28	0.22	0.47	0.03	8.4	0.53
S1	0.42	0.09	1.87	0.14	0.88	0.09	8.59	0.91
S2	0.42	0.08	2.36	0.19	0.67	0.11	4.02	0.62
S3	1.13	0.32	3.94	0.85	0.74	0.18	3.07	0.65

Abbreviations: GSTM1-glutathione S-transferases M1; HR-hazard ratio; CI-confidence interval; IGCCCG-International Germ Cell Collaborative Group; Note: [a]low QS (QS<10) vs. high QS (QS≥10)

GSTM1 variant detection. GSTM1 variant detection led to the identification of 0-4 SNVs per cell line, all classified as benign according to the ACMG guidelines (as of April 2024). Any short in/dels were detected in GSTM1. In terms of CNVs, the NTERA-2 parental cell line was without CNVs, while the resistant version had duplicated exons 5 and 7 of the GSTM1 with 6 extra copies each. TCam-2, both parental and resistant, were without CNVs. The JAR parental cell line had a duplicated exon 7 with 37 extra copies of it, while the resistant version was without CNVs. Both NOY-1 cell lines, parental and resistant, had a homozygous deletion of the whole GSTM1. Finally, the JEG-3 parental cell line had a duplicated exon 7 with 25 extra copies, while the resistant version had duplicated exons 3, 5, 6 and 7 with 2 extra copies each.

Gstm1 IHC in GCTs xenografts. The mean QS±SD of GSTM1 evaluated by immunohistochemistry in GCTs cell line xenografts was 7.60±4.40, with the highest level of JEG-3

and NOY-1 with no difference in QS between cisplatin-sensitive and their resistant variants (p=0.91; Supplementary Table S6).

GSTM1 expression in publicly available databases. The expression of GSTM1 gene can be detected across all types of human cancers including TGCT, however the data from normal testis tissue is not available in the the Cancer Genome Atlas (TCGA). When transcription of the GSTM1 gene in the GTEx Testis cohort was compared to the TCGA TGCT cohort, it indicated significantly lower GSTM1 expression in TGCT tumors. Data from the Cancer Cell Line Encyclopedia (CCLE) confirmed expression of GSTM1 in NTERA2CLD1, TERA2 (embryonal carcinoma) and SUSA (teratoma) cell lines. No expression is detected in 1777NRPMET, NCCIT and 1618K (embryonal carcinoma) (Figure 4).

Table 4. Multivariate analysis of factors associated with progression-free survival.

Variable	HR	Lower 95% CI	Upper 95% CI	p-value
Gstm1				
high vs. low	2.03	1.06	3.90	0.0332
IGCCCG risk group				
Intermediate vs. good	4.19	1.86	9.41	0.0005
poor vs. good	8.58	4.36	16.89	<0.0001

Abbreviations: GSTM1-glutathione S-transferases M1; HR-hazard ratio; CI-confidence interval; IGCCCG-International Germ Cell Collaborative Group

Discussion

In this translational study, the association between GSTM1 level in GCTs and patient outcome was observed for the first time. Patients with a low GSTM1 level in tumor tissue had a better PFS compared with high GSTM1 level. This association was most striking for the IGCCCG good prognostic group, where G GSTM1 was consistently associated with PFS and OS. In most subgroups, low GSTM1 level was found to be correlated with good prognosis. However, the lack of prognostic value could be due to the small sample size with consequent low statistical power to detect differences. Surprisingly, we didn not notice any association between GSTM1 and patient/tumor characteristics, except

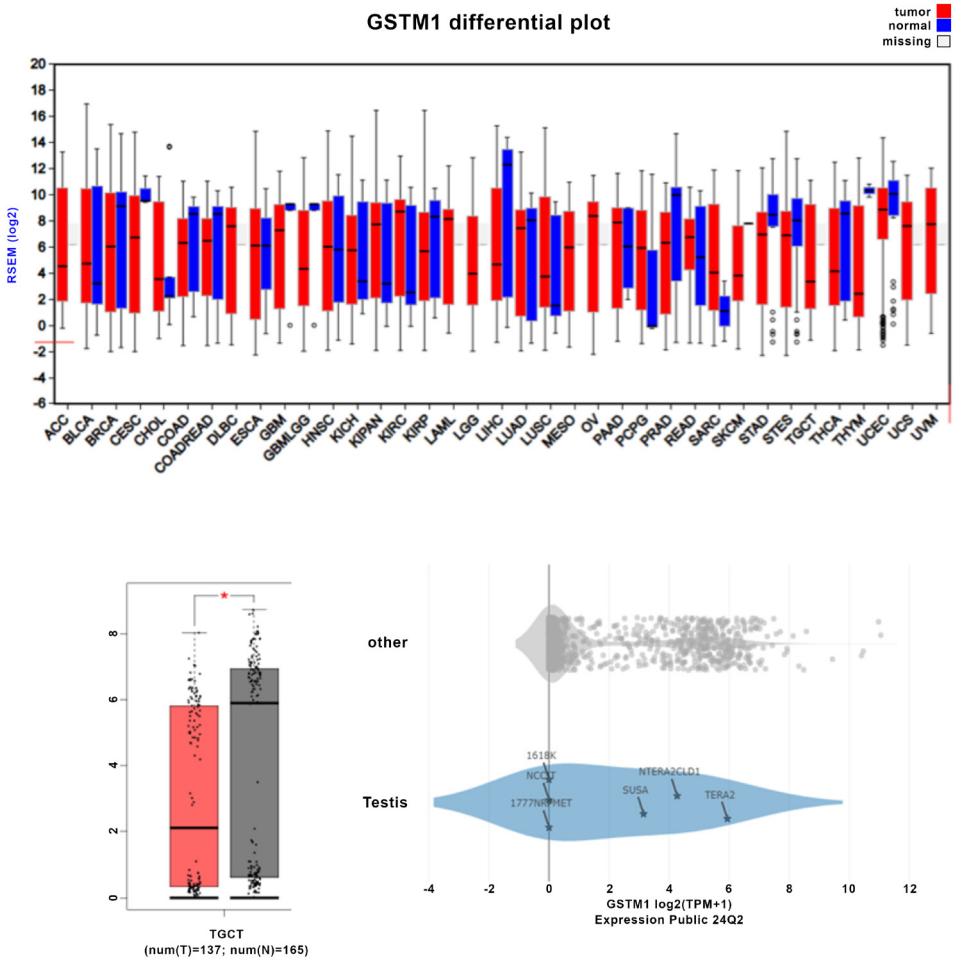

Figure 4. GSTM1 expression data from publicly available resources. A) GSTM1 differential plot illustrating the expression of GSTM1 gene across human cancers including TGCT, the data from normal testis tissue is not available. Data from the Cancer Genome Atlas (TCGA) were analyzed using FireBrowse portal of the Broad Institute (MIT, Harvard, www.firebrowse.org). B) Transcription of GSTM1 gene in GTEx Testis cohort was compared to TCGA TGCT-analysis was performed by UCSC Xena browser and indicated significantly lower GSTM1 expression in TGCT tumors. The Gene Expression Profiling Interactive Analysis database was used for visualisation [43] C) Expression of GSTM1 gene in non-seminomatous TGCT cell lines in the dataset Expression 24Q2 from the Cancer Cell Line Encyclopedia was plotted according to the tumor type. Expression was detected in NTERA2CLD1, TERA2 (embryonal carcinoma) and SUSA (teratoma) cell lines. No expression is detected in 1777NRPMET, NCCIT and 1618K (embryonal carcinoma). Upper panel in grey depicts the distribution of GSTM1 expression in the other cancer cell lines [44].

its association with histological subtype, with the highest expression in seminoma and embryonal carcinoma, and the lowest in teratoma. These results suggested that Gstm1 level could influence treatment outcomes but is not directly involved in the pathogenesis of GCTs.

In vitro and *in vivo* analysis using GCT cell line xenografts did not reveal an association between *GSTM1* expression and cisplatin resistance. Furthermore, there was a lack of correlation between the gene expression results and protein expression, specifically that of JEG-3 and NOY-1. When comparing the expression in cells with that in their corresponding xenografts, an increase was noted in the xenograft expression, indicating a change in gene expression and upregulation *in vivo*. In addition, in some of the GCTs cell lines, even cisplatin-resistant ones, very low or no *GSTM1* expression was observed. WES revealed homozygous deletion of whole *GSTM1* in parenteral NOY-1 cell lines and their cisplatin-resistant variant, and this was consistent with the absence of *GSTM1* expression noticed both *in vitro* and in the xenograft model [24]. It was shown that a significant percentage of normal individuals exhibited a common polymorphism characterized by the complete deletion of the *GSTM1* gene (null allele. Numerous studies showed a correlation between this *GSTM1* polymorhism and treatment outcomes in different types of cancer, while data evaluating *GSTM1* expression in tumor tissue were limited [19, 21, 22]. However, *GSTM1* expression was influenced not only by its genotype but also by other factors. For example, physical activity could affect their expression [35]. Furthermore, emerging data suggested that *GSTM1* expression is regulated by ncRNAs, microRNAs and epigenetically [36–38].

Data regarding the relationship between GSTM1 level in normal and tumor tissue was lacking. However, it was suggested that *GSTM1* expression in tumor cells is more important in treatment resistance than its expression in normal tissue. We hypothesized that the lack of prognostic value of GSTM1 in the poor risk subgroup, as well as the lack of correlation *in vitro* and in the xenograft model, could be due to different mechanisms of resistance in various GCTs models, and could be distinct in different groups of patients. This could be the case, especially for teratoma and/or yolk sac tumors that are mostly resistant to cisplatin-based therapy, but GSTM1 when translating treatment results from pre-clinical models to the clinic. Recently, it was shown that the gain of chromosome 3p25.3 is associated with cisplatin resistance in a group of patients and is an independent predictor of poor outcomes in male GCTs [39]. Beyond, other supposed mechanism of resistance in GCTs this suggest, there is probably not one common mechanism of treatment resistance that could be responsible for treatment failure [8]. Furthermore, other GSTs, such as GSTM3 and glutathione S-transferase theta-1, that were not evaluated in this patient cohort could also affect the treatment outcome.

GSTM1 is an enzyme that is involved in the detoxification of reactive oxygen species, and its role in cisplatin resistance has been shown in different types of cancer [10]. It is hypothesized that GSTM1 plays a role in cisplatin resistance, mainly in tumors of endodermal origin, where the nuclear factor erythroid 2-related factor 2 pathway is activated. However, GCTs are of mesodermal origin, and in this setting, DNA repair and decreased apoptosis induction are more important in cisplatin resistance [40]. As GSTM1 also has a non-enzymatical activity that could play a role in treatment resistance, it can not be determined which of these mechanisms influence the treatment outcome more. The *GSTM1* gene exhibits various SNVs that are associated with different clinical activity and as was shown also with treatment toxicity [23, 24]. GST inhibitors could overcome resistance of cancer cells to antitumor drugs and thus there is biological rationale for GST inhibition in cancer therapy. Most established and newly synthesized GST inhibitors have been primarily evaluated for their cytotoxic effects in cancer cell lines and mouse xenograft models, demonstrating promising activities. However, a significant issue has emerged: the lack of specificity of these inhibitors when tested in vitro for their inhibition properties [9]. A number of GST inhibitors like curcumin or ethacrynic acid and its analogous have been identified and evaluated in the preclinical setting and have already entered the early phase of clinical drug development [9, 41, 42]. We hypothesized that GSTM1 inhibition during platinum-based chemotherapy could lead to better outcomes.

All patients in the present study were chemo-naive and none were platinum-resistant/refractory. Therefore, the role of GSTM1 in treatment resistance in relapsed GCTs could not be determined. We hypothesized that GSTM1 is a factor that modifies treatment outcomes in GCTs; however, other mechanisms are also involved in treatment resistance, particularly in the poor prognosis subgroup.

The study limitations include a moderate sample size and low proportion of certain subgroups, such as extragonadal GCTs, or patients with visceral non-pulmonary metastases. Another limitation represents GSTM1 quantification that performed by IHC and was semiquantitative. We also can't exclude the effect of the difference in archival time of FFPE that varies between 1999 and 2013 on the stability of the GSTM1 epitope that were detected by monoclonal antibody utilized in this study. The heterogeneity of GCTs and tissue microarray that did not show the high volume of GCT components could be another limitation. Another study limitation was the absence of tumor tissue following chemotherapy in refractory GCTs. Analysis of these materials could better define the role of GSTM1 in this clinical setting. We performed WES of all utilized cell lines to get the data related to CNV of *GSTM1*. Due to limited amount of FFPE, we are not able to perform this analysis from patients' samples, that represent another limitation of the study. However, our hypothesis was that GSTM1 level in tumor tissue is associated with patient's outcome due to inactivation of cisplatin, and we suggest that results of genetic analysis of *GSTM1* gene in FFPE don't impact this hypothesis. Genetic analysis

just shows one of the mechanism that affect *GSTM1* expression but doesn't affect the primary hypothesis, as other mechanism beyond CNV could affect *GSTM1* expression. In patients with homozygous deletion of GSTM1 and subsequent absence of *GSTM1* expression we suggest that other mechanism of cisplatin inactivation including other GSTs could play a role in mechanism of resistance.

In conclusion, the present study was the first to show that the tumor level of GSTM1 is prognostic in GCTs, with a high GSTM1 level was associated with worse outcomes. GSTM1 could be responsible, in part, for treatment resistance in GCTs, and GSTM1 inhibition during platinum-based chemotherapy could lead to better outcomes, at least for a subgroup of patients.

Supplementary information is available in the online version of the paper.

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Association between glutathione S-transferases M1 expression and treatment outcome in germ cell tumor patients

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Supplementary Information

Supplementary Table S1. Patient characteristics according to histology.

			Seminoma		Non-seminoma	
	N	%	N	%	N	%
All	207	100.0	37	100.0	170	100.0
Histology						
seminoma	37	17.9	37	100.0	0	0.0
non-seminoma	170	82.1	0	0.0	170	100.0
Tumor primary						
gonadal	200	96.6	37	100.0	163	95.9
retroperitoneal	5	2.4	0	0.0	5	2.9
mediastinal	2	1.0	0	0.0	2	1.2
Staging						
metastatic	169	81.6	37	100.0	132	77.6
non-metastatic	38	18.4	0	0.0	38	22.4
Sites of metastases						
retroperitoneum	147	71.0	35	94.6	112	65.9
mediastinum	18	8.7	3	8.1	15	8.8
lungs	49	23.7	5	13.5	44	25.9
liver	11	5.3	0	0.0	11	6.5
brain	2	1.0	0	0.0	2	1.2
visceral non-pulmonary metastases	14	6.8	1	2.7	13	7.6
No of metastatic sites						0.0
0	52	25.1	0	0.0	52	30.6
1 to 2	128	61.8	35	94.6	93	54.7
≥3	27	13.0	2	5.4	25	14.7
IGCCCG risk group						
good prognosis	117	56.5	33	89.2	84	49.4
intermediate prognosis	23	11.1	4	10.8	19	11.2
poor prognosis	29	14.0	0	0.0	29	17.1

Supplementary Table S2. Expression of GSTM1 according to histology.

Histologic Subtype	Nr. of samples (N)	Mean QS	Median	SD	SEM
germ cell tumors[a]	207	5.48	4.00	7.76	0.54
embryonal carcinoma	124	3.60	3.00	3.46	0.28
choriocarcinoma	12	6.42	5.00	5.55	1.04
seminoma	77	7.06	6.00	4.55	0.41
teratoma	50	1.16	0.00	2.08	0.51
yolc sac tumor	32	3.56	2.00	3.68	0.64

Notes: [a]highest GSTM1 expression in any histological subtype in case of mixed germ cell tumour (N=63)

Supplementary Table S3. Association between GTSM1 and patients/tumor characteristics in seminoma.

	Seminoma					
	N	**Mean**	**Median**	**SD**	**SEM**	**p-value**
All						
Histology						
seminoma	37	5.89	6.00	4.07	1.28	0.11
non-seminoma	170	5.39	4.00	8.35	0.60	
Tumor primary						
gonadal	37	5.89	6.00	4.07	1.28	NA
retroperitoneal	0					
mediastinal	0					
Staging						
metastatic	37	5.89	6.00	4.07	1.28	NA
non-metastatic	0					
Sites of metastases						
retroperitoneum						
no	2	4.50	4.50	0.71	2.86	0.53
yes	35	6.15	6.00	4.11	0.69	
mediastinum						
no	34	6.00	6.00	3.93	0.70	0.89
yes	3	7.00	7.00	7.07	2.87	
lungs						
no	32	5.77	5.00	4.00	0.72	0.24
yes	5	7.80	6.00	4.02	1.79	
liver						
no	37	NA				NA
yes	0					
brain						
no	37	NA				NA
yes	0					
visceral non-pulmonary metastases						
no	36	6.11	6.00	4.05	0.68	0.56
yes	1	4.00	4.00		4.05	
No of metastatic sites						
0	0	0.00	0.00	0.00	0.00	0.25
1 to 2	35	5.88	5.50	3.99	0.69	
≥3	2	9.00	9.00	4.24	2.83	
IGCCCG risk group						
good	33	5.75	5.50	3.95	0.70	0.22
intermediate	4	8.50	9.00	4.12	1.98	
poor	0					

Supplementary Table S4. Association between GTSM1 and patients/tumor characteristics in non-seminoma.

	Non-seminoma					
	N	**Mean**	**Median**	**SD**	**SEM**	**p-value**
All						
Histology						
seminoma	37	5.89	6.00	4.07	1.28	0.11
non-seminoma	170	5.39	4.00	8.35	0.60	
Tumor primary						
gonadal	163	5.42	4.00	8.51	0.66	0.48
retroperitoneal	5	3.40	3.00	1.67	3.75	
mediastinal	2	7.50	7.50	3.54	5.93	
Staging						
metastatic	38	4.61	4.00	3.89	1.36	0.67
non-metastatic	132	5.61	4.00	9.24	0.73	
Sites of metastases						
retroperitoneum						
no	58	4.62	4.00	3.84	1.10	0.62
yes	112	5.79	4.00	9.90	0.79	
mediastinum						
no	155	5.50	4.00	8.65	0.67	0.43
yes	15	4.27	3.00	4.13	2.16	
lungs						
no	126	5.75	4.00	9.38	0.74	0.30
yes	44	4.36	3.50	4.05	1.26	
liver						
no	159	5.48	4.00	8.54	0.66	0.25
yes	11	4.00	2.00	4.69	2.52	
brain						
no	168	5.36	4.00	8.39	0.65	0.17
yes	2	8.00	8.00	2.83	5.92	
visceral non-pulmonary metastases						
no	157	5.45	4.00	8.60	0.67	0.72
yes	13	4.62	3.00	4.31	2.32	
No of metastatic sites						
0	52	4.52	4.00	3.84	1.16	0.34
1 to 2	93	6.17	5.00	10.67	0.87	
≥3	25	4.28	3.00	4.21	1.67	
IGCCCG risk group						
good	84	6.08	4.00	11.16	1.01	0.22
intermediate	19	5.71	5.00	4.22	2.02	
poor	29	4.07	2.00	3.99	1.79	

Supplementary Table S5. Overview of the xenografts used for GSTM1 expression analysis and IHC.

Cells injected	Duration of xenograft growth (days)	Xenograft volume (mm^3)	Sample processing
NTERA-2 (EC)	21	320	FFPE
NTERA-2 (EC)	21	547	RNA
NTERA-2 CisR (EC)	28	395	FFPE
NTERA-2 CisR (EC)	28	517	RNA
TCam-2 (SE)	63	545	FFPE
TCam-2 (SE)	63	362	RNA
TCam-2 CisR (SE)	15	719	FFPE
TCam-2 CisR (SE)	15	265	RNA
JAR (ChC)	60	1095	FFPE
JAR (ChC)	60	340	RNA
JAR CisR (ChC)	50	412	FFPE
JAR CisR (ChC)	50	423	RNA
JEG-3 (ChC)	12	294	FFPE
JEG-3 (ChC)	12	266	RNA
JEG-3 CisR (ChC)	17	492	FFPE
JEG-3 CisR (ChC)	17	437	RNA
NOY-1 (YST)	45	261	FFPE
NOY-1 (YST)	45	301	RNA
NOY-1 CisR (YST)	24	221	FFPE
NOY-1 CisR (YST)	24	791	RNA

Supplementary Table S6. Expression of GSTM1 according to histology in mouse xenograft model.

Group	QS parental	High QS (QS≥10)	QS cisplatin resistant variant	High QS (QS≥10)
TCam-2	2	no	8	no
NTERA-2	6	no	0	no
JEG-3	12	yes	12	yes
JAR	6	no	6	no
NOY-1	12	yes	12	yes
All cell lines (median)	6	2	8	2