Genetic Determinants of Cisplatin Resistance in Patients With Advanced Germ Cell Tumors

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ABSTRACT

Purpose

Owing to its exquisite chemotherapy sensitivity, most patients with metastatic germ cell tumors (GCTs) are cured with cisplatin-based chemotherapy. However, up to 30% of patients with advanced GCT exhibit cisplatin resistance, which requires intensive salvage treatment, and have a 50% risk of cancer-related death. To identify a genetic basis for cisplatin resistance, we performed whole-exome and targeted sequencing of cisplatin-sensitive and cisplatin-resistant GCTs.

Methods

Men with GCT who received a cisplatin-containing chemotherapy regimen and had available tumor tissue were eligible to participate in this study. Whole-exome sequencing or targeted exon-capture-based sequencing was performed on 180 tumors. Patients were categorized as cisplatin sensitive or cisplatin resistant by using a combination of postchemotherapy parameters, including serum tumor marker levels, radiology, and pathology at surgical resection of residual disease.

Results

TP53 alterations were present exclusively in cisplatin-resistant tumors and were particularly prevalent among primary mediastinal nonseminomas (72%). *TP53* pathway alterations including *MDM2* amplifications were more common among patients with adverse clinical features, categorized as poor risk according to the International Germ Cell Cancer Collaborative Group (IGCCCG) model. Despite this association, *TP53* and *MDM2* alterations predicted adverse prognosis independent of the IGCCCG model. Actionable alterations, including novel *RAC1* mutations, were detected in 55% of cisplatin-resistant GCTs.

Conclusion

In GCT, *TP53* and *MDM2* alterations were associated with cisplatin resistance and inferior outcomes, independent of the IGCCCG model. The finding of frequent *TP53* alterations among mediastinal primary nonseminomas may explain the more frequent chemoresistance observed with this tumor subtype. A substantial portion of cisplatin-resistant GCTs harbor actionable alterations, which might respond to targeted therapies. Genomic profiling of patients with advanced GCT could improve current risk stratification and identify novel therapeutic approaches for patients with cisplatin-resistant disease.

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INTRODUCTION

Germ cell tumors (GCTs) are the most common solid tumors diagnosed in men 15 to 40 years old.¹ Most patients with metastatic GCT are cured with cisplatin-based chemotherapy combinations, which is unique among adult solid tumors. However, 20% to 30% of those with advanced disease progress after first-line chemotherapy and

require intensive salvage regimens, which include high-dose chemotherapy with autologous stem cell transplantation and desperation surgery. Despite such efforts, nearly half of these patients are destined to die of progressive GCT.² The clinically based International Germ Cell Cancer Collaborative Group (IGCCCG) prognostic model stratifies patients into good-, intermediate-, and poor-risk groups. Understanding the molecular and genetic pathogenesis of the disease,

including determinants of cisplatin sensitivity and resistance, could allow for enhanced risk stratification and more accurate identification of high-risk patients most in need of novel therapeutic approaches. Prior investigations have yielded conflicting results regarding the association of genomic alterations with cisplatin resistance in GCT.^{3,4} In addition, the utility of prior studies was limited by small patient populations, the inclusion of few patients with cisplatin-resistant disease, and/or the lack of comprehensive sequencing approaches.

With the goal of identifying recurrent genetic alterations associated with cisplatin resistance in GCT, we performed whole-exome sequencing (WES) on a discovery cohort of 19 tumors and validated our findings using selective exon-capture sequencing of an additional 161 GCTs enriched for the cisplatin-resistant phenotype.

PATIENTS AND METHODS

Patient Eligibility

This study was conducted following institutional review board approval. All specimens were obtained from patients evaluated at Memorial Sloan Kettering Cancer Center. Men who received standard first-line cisplatin-based chemotherapy for advanced GCT of any primary site were eligible if they provided informed consent for tumor molecular characterization and had available histologically confirmed fresh or archived tumor tissue containing viable GCT and matching normal DNA. Pure teratoma and pure malignant transformation tumors were excluded. Standard first-line chemotherapy consisted of at least three cycles of a cisplatin-based combination regimen, typically etoposide plus cisplatin (EP), bleomycin plus EP (BEP), or ifosfamide plus EP. First-line treatment with paclitaxel plus ifosfamide plus cisplatin as part of a phase II clinical trial (NCT01873326) was also allowed. Patients who received only adjuvant chemotherapy, carboplatin-based regimens, or fewer than three cycles of cisplatin-based chemotherapy were ineligible. Nonprogressing patients were required to have at least 1 year of clinical follow-up.

Definitions of Response and Cisplatin Sensitivity and Resistance

A complete response (CR) to first-line cisplatin-based chemotherapy (CR chemo) was defined as tumor marker normalization and either complete resolution of all radiographic evidence of tumor masses or complete surgical resection of residual tumor masses revealing necrosis and/or teratoma. When complete surgical resection revealed viable nonteratomatous GCT elements, it was termed a CR to chemotherapy plus surgery (CR chemo + surgery). Patients who achieved marker normalization but did not undergo complete surgical resection of residual disease were considered to have achieved a partial response with negative tumor markers (PR-negative markers). If such patients underwent partial tumor resection, the pathology could consist of only teratoma or necrosis but no viable GCT elements. All other patients were categorized as having an incomplete response (IR).

Cisplatin resistance has a more stringent definition in GCT than other adult solid tumors. In this study, patients with cisplatin resistance had to meet one of the following criteria: (1) IR to first-line cisplatin-based chemotherapy, (2) nonteratomatous tumor progression after first-line cisplatin-based chemotherapy, or (3) viable nonteratomatous GCT identified at postchemotherapy surgery. All other patients were considered cisplatin-sensitive.

Next-Generation Sequencing

High-quality, fresh-frozen tumor tissues were selected for WES analysis from a discovery cohort consisting of 19 specimens. Representative

hematoxylin-and-eosin-stained sections were reviewed by a Memorial Sloan Kettering Cancer Center genitourinary pathologist and were confirmed to have > 40% tumor purity. Additional details regarding the WES specimens and sequencing are provided in the methods section of the Appendix (online only).

Targeted, capture-based, next-generation sequencing of more than 300 cancer-related genes to a mean depth of 500- to 1,000-fold coverage was performed using the Memorial Sloan Kettering Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) assay. MSK-IMPACT was performed on the 19 WES tumors to confirm alterations identified by WES and to improve the sensitivity in detecting known cancer-associated genetic alterations.⁵ MSK-IMPACT was also performed on tumors from a prospective validation cohort that comprised 161 additional patients with advanced GCT. Following DNA extraction from tumor- and patient-matched normal samples, barcoded libraries were captured, sequenced, and analyzed through a customized pipeline for somatic mutation calling, copy number alterations, and structural rearrangements, as previously described.³

Functional Assays

Details of the methods used to functionally characterize *RAC1* mutations are provided in the Appendix (online only).

Statistical Analysis

We assessed the association between alterations and clinicopathologic features (ie, histology, primary site, IGCCCG risk group, sample location) using Fisher's exact test. Genes enriched in cisplatin-resistant versus cisplatin-sensitive tumors in the discovery cohort were examined for association with cisplatin-sensitivity in the validation cohort. Given the relative paucity of recurrent mutations, the entire cohort (discovery plus validation; N = 180) was used to assess for associations between genetic alterations and clinicopathologic features.

All clinical data were collected through retrospective chart review. Progression-free survival (PFS) was calculated from start date of chemotherapy to date of progression or death. Cox regression was used to evaluate multivariable associations with PFS.

RESULTS

Table 1 depicts patient and tumor characteristics for the combined cohort (N = 180), including the 19-sample discovery set and the 161-sample validation set. In the discovery cohort, nine tumors were cisplatin sensitive and 10 were resistant. Of the resistant tumors, eight were metastatic lesions and eight were collected after administration of chemotherapy. By definition, we were not able to obtain cisplatin-sensitive tumors after chemotherapy. WES was performed on all 19 samples with mean sample coverage of 116× (range, 93× to 134×) with 90% of target bases covered at greater than 30×. The mean number of somatic mutations per sample was 44 (standard deviation [SD], 50; median, 22; range, 10 to 208) and the mean number of nonsynonymous mutations was 31 (SD, 33; median, 16; range, 7 to 137; Appendix **Table A1**). The mean rate of 0.9 mutations per megabase with MSK-IMPACT was very low compared with other adult solid tumors.⁶ The median number of total (46 v 21; *P* = .05) and nonsynonymous (36 v 14; *P* = .04) mutations was higher among cisplatin-resistant tumors than among cisplatin-sensitive tumors (**Fig 1A**).

As expected, evidence of 12p gain was noted in most tumors (74%) in the discovery cohort, but few other recurrent alterations were identified (**Fig 1B**). Notably, among 10 patients with cisplatin-

Table 1. Patient Characteristics (discovery and validation cohorts)

Variable	All Patients (N = 180)	Sensitive (n = 76)	Resistant (n = 104)
Median age, years (range)	30.6 (16-65.1)	31.2 (16-65.1)	29.8 (18.2-59.2)
Sample histology			
Nonseminoma	126 (70)	50 (65.8)	76 (73.1)
Seminoma	54 (30)	26 (34.2)	28 (26.9)
Primary site			
Testis	157 (87.2)	70 (92.1)	87 (83.7)
Mediastinum	22 (12.2)	5 (6.6)	17 (16.3)
Retroperitoneum	1 (0.6)	1 (1.3)	0 (0)
IGCCCG			
Good	92 (51.1)	53 (69.7)	39 (37.5)
Intermediate	28 (15.6)	15 (19.7)	13 (12.5)
Poor	59 (32.8)	8 (10.5)	51 (49)
Unknown	1 (0.6)	0 (0)	1 (1)
Initial chemotherapy			
BEP	67 (37.2)	15 (19.7)	52 (50)
EP	76 (42.2)	48 (63.2)	28 (26.9)
TIP or VIP	36 (20)	13 (17.1)	23 (22.1)
PVB	1 (0.6)	0 (0)	1 (1)
Sample type			
Primary	108 (60)	64 (84.2)	44 (42.3)
Metastasis	72 (40)	12 (15.8)	60 (57.7)
Sample collection			
Prechemotherapy	113 (62.8)	76 (100)	37 (35.6)
Postchemotherapy	67 (37.2)*	0 (0)	67 (64.4)*
Died of disease	24 (13.3)	0 (0)	24 (23.1)

NOTE: Data given as no. (%) unless otherwise indicated.

Abbreviations: BEP, bleomycin, etoposide, and cisplatin; EP, etoposide and cisplatin; IGCCCG, International Germ Cell Cancer Collaborative Group; PVB, cisplatin, vinblastine, and bleomycin; TIP, paclitaxel, ifosfamide, and cisplatin; VIP, VP-16, ifosfamide, and cisplatin.

*One patient with mediastinal primary received one cycle of BEP chemotherapy before sample acquisition for sequencing.

resistant disease, two had *TP53* alterations (one mutation [V173M] and one intragenic deletion) and three had focal amplification of *MDM2*, an E3 ubiquitin-protein ligase that negatively regulates *TP53*. Thus, five of 10 cisplatin-resistant tumors harbored alterations within the *TP53/MDM2* pathway compared with none of the cisplatin-sensitive tumors ($P = .033$; Fig 1B).

To further explore this finding and mitigate potential bias associated with differences in sample timing relative to chemotherapy (ie, before v after) and sample type (primary v metastatic) between the cisplatin-sensitive and cisplatin-resistant cohorts, we performed MSK-IMPACT sequencing on a prospective cohort of 161 patients with GCT.⁵ In this validation cohort, 102 specimens

(63%) were acquired before chemotherapy and 64 (40%) were metastatic lesions. An additional 14 hotspot mutations and one homozygous deletion of *TP53* were identified. *TP53* alterations were found exclusively in tumors from patients with cisplatin-resistant disease. Seven additional tumors in the validation set harbored *MDM2* amplifications. As in other tumor types, amplification of *MDM2* was mutually exclusive with *TP53* alteration. The majority of patients with *MDM2* amplification (five of seven [71.4%]) had cisplatin-resistant disease. Notably, alterations within *TP53* and *MDM2* were significantly more common in patients with cisplatin-resistant versus cisplatin-sensitive GCT (21.3% v 3%; $P = .001$).

Fig 1. Differences between cisplatin-sensitive and cisplatin-resistant germ cell tumors (GCTs) in the discovery set. (A) Frequency of nonsynonymous mutations identified on whole-exome sequencing was higher among cisplatin-resistant than cisplatin-sensitive GCTs (36 [range, 8-137] v 14 [range, 7-28]; $P = .04$). (B) OncoPrint displaying the distribution of alterations in *TP53*, *MDM2*, *KRAS*, and *NRAS* in patients with cisplatin sensitivity and those with cisplatin resistance, as analyzed by whole-exome sequencing. Each column represents an individual patient or sample. Distribution of 12p gain among the discovery set samples is also shown.

We used the combined discovery and validation datasets to evaluate for associations between recurrent genetic alterations and clinicopathologic features (Table 1; Fig 2A). In the combined dataset, *TP53* mutations/deletions alone and in combination with *MDM2* amplifications were significantly more common in resistant versus sensitive tumors (*TP53*, 17 of 104 [16.3%] ν none of 76 [0%], $P < .001$; *TP53/MDM2*, 25 of 104 [24.0%] ν two of 76 [2.6%], $P < .001$; Fig 2B). When considered alone, there was a trend toward more frequent *MDM2* amplification in resistant tumors (eight of 104 [7.6%] ν two of 76 [2.6%]; $P = .195$). Figure 3A illustrates the distribution of *TP53* mutations, which included previously reported recurrent missense mutations and truncations. With a median follow-up of 38.8 months, patients with *TP53/MDM2* pathway alterations had significantly shorter PFS than those without these alterations (Fig 4). In addition, *TP53/MDM2* alterations were more frequent among patients in the IGCCCG poor-risk group as compared with those in the intermediate- and good-risk groups (32.2% ν 7.1% ν 6.5%; $P < .001$). In a multivariable analysis that included the IGCCCG

risk model, the presence of a *TP53/MDM2* alteration was an independent predictor of disease progression following first-line cisplatin-based chemotherapy (hazard ratio, 1.83; 95% CI, 1.12 to 2.98; $P = .016$).

We also identified a strikingly higher rate of *TP53* alterations in samples from patients with a mediastinal versus a testicular primary tumor site (13 of 22 [59.1%] ν four of 158 [2.5%]; $P < .001$). When limited to primary mediastinal nonseminoma, which has a distinctly more aggressive biology and unfavorable outcome than primary mediastinal seminoma, the alteration rate was even more pronounced (13 of 18 [72.2%]) because none of the four primary mediastinal seminomas harbored a *TP53* mutation.⁷

An intact *TP53* pathway that facilitates apoptosis has been postulated to explain the exquisite cisplatin sensitivity characteristic of most GCTs.⁸ We thus used a pathway-based analysis to identify additional recurrent alterations predicted to impact *TP53*-mediated downstream functions and assessed their association with cisplatin sensitivity. Among *TP53*-associated genes, *MYCN* amplifications were identified in five patients (all cisplatin

Fig 2. Genomic alterations among 180 germ cell tumor (GCT) samples in the combined discovery and validation cohorts. (A) OncoPrint displaying the most frequently altered and other biologically notable mutations. (B) *TP53* alterations and combined *MDM2/TP53* alterations were more common among resistant GCT samples than sensitive ones. Indel, insertion/deletion.

Fig 3. Distribution of gene mutations among 180 germ cell tumor (GCT) samples in the combined cohort. (A) *TP53* mutations. (B) *KIT* mutations. The distribution of *KIT* mutations in GCT differs from GI stromal tumors. (C) Alignment and number of *KRAS* and *RAC1* mutations at the 12, 34, and 61 positions. Expression of the *RAC1* mutations identified in GCT results in increased phosphorylation of PAK1 and ERK. Ig, immunoglobulin.

resistant), four of whom had *TP53*/*MDM2* wild-type tumors (Fig 2A). *MYCN*, which transcriptionally targets both *TP53* and *MDM2*, is a marker of poor prognosis in neuroblastoma, in which *TP53* mutations are also rare (< 2%).

We grouped all other somatic mutations into core signal transduction pathways or canonical cell functions with the goal of identifying additional genes predictive of cisplatin resistance and/or potential targets for therapy, using a precision medicine approach. Potentially actionable alterations ($n = 75$) were identified in 57 of 104 patients (55%) with cisplatin-resistant disease (Appendix Table A2, online only). Within the receptor tyrosine kinase (RTK) pathway, we identified 20 hotspot mutations in *KIT* among 19 patients. In contrast to GI stromal tumors, most of these were localized to exon 17 and are associated with imatinib resistance (Fig 3B).⁹ *KIT* mutations were also more common in seminoma than nonseminoma (16 of 54 [29.6%] ν five of 126 [4%]; $P < .001$). Seven additional tumors contained alterations in *CBL*, a negative regulator of *KIT*, including one previously identified hotspot mutation (W408R) in the RING finger domain, three X410 splice sites, and one homozygous deletion.^{10,11} One activating mutation in *GNAQ*

(Q209P), a common finding in ocular melanoma,¹² was also identified. Four patients had amplifications in *KDR* and one had an amplification of *MET*. Potential therapies directed against each of these alterations are provided in Appendix Table A2 (online only).

RAS/ERK pathway mutations were identified in 33 patients (Fig 2A), including 23 hotspot mutations in *KRAS* in 22 patients (15 at the G12 codon) along with four *NRAS* mutations (Fig 2A). *KRAS* mutations were significantly more frequent in seminomas versus nonseminomas (11 of 54 [20%] ν 11 of 126 [8.7%]; $P = .045$). As previously observed,³ the majority of *KRAS* mutations in nonseminoma (eight of 11 [72.7%]) were present in cisplatin-resistant tumors, as were three of four mutations in *NRAS*. In contrast, no association between RAS mutation and cisplatin sensitivity was noted for seminoma.³

In keeping with prior work, three hotspot *BRAF* mutations were also identified⁴ (D594N, D594G, and G466E) and the rate of *BRAF* mutations within the entire cohort (1.7%) and among patients with cisplatin resistance (2.9%) was low.^{3,4} Nine mutations were identified in *RAC1* (G12V, $n = 3$; G12R, $n = 2$; P34R, Q61R, and Q61K, $n = 2$; Fig 3C), a member of the Rho family of GTPases, with significant homology to the RAS family, including

Fig 3. (Continued).

conserved residues at positions 12, 34, and 61.¹³ The *RAC1* mutants identified in the GCT cohort were expressed within HEK293 cells, confirming that each was associated with increased expression of phosphorylated PAK1 and phosphorylated MEK (Fig 3C).

Finally, consistent with prior data, 24 samples (13.3%) had mutually exclusive phosphoinositide 3-kinase (PI3K) pathway mutations, including four *PIK3CA* E542K mutations, five loss-of-function mutations in *PTEN* (V85F, Q149K, S361N, and Y178 deletion), one *AKT1* amplification, two *MTOR* mutations (T1977K and A1513P), two putative loss of function alterations in *TSC1* (F216 frameshift and homozygous deletion), and four novel *TSC2* mutations (Fig 2).³ Other notable findings included inactivating mutations or deletions in *APC* (n = 3); *FAT1* (n = 4); *AXIN1* (n = 2); *EP300*, *SETD2*, and *PTPRD* (n = 3); and *BRCA2* tumor suppressor genes. All mutational and clinical data are available on the cBio Cancer Genomics Portal.¹⁴

DISCUSSION

To our knowledge, ours is the largest series reported to date of advanced GCT analyzed with massively parallel next generation

sequencing. We identified *TP53* pathway alterations among a significant proportion of patients with cisplatin-resistant disease. In particular, *TP53* alterations were present exclusively in patients with cisplatin-resistant tumors (Figs 2A and 2B). In addition, as one might expect for a biomarker of adverse outcome, mutually exclusive *TP53/MDM2* alterations were significantly more frequent among patients with unfavorable clinical characteristics, such as categorization in the IGCCCG poor-risk group and having a mediastinal primary tumor site. Although mediastinal origin has no effect on prognosis for seminoma (characterized by chemosensitivity and excellent outcomes), for nonseminoma, it singularly confers poor-risk classification in the IGCCCG model. Furthermore, within the IGCCCG poor-risk group, having a mediastinal primary tumor site is considered the most unfavorable characteristic as it is associated with the lowest rates of PFS and overall survival.⁷ Thus, to our knowledge, the provocative data in this series provide, for the first time, a genetic and biologic basis for the unique and adverse clinical phenotype of these tumors as compared with gonadal nonseminoma and mediastinal seminoma. Furthermore, the association of *TP53* pathway alterations with shorter PFS independent of the IGCCCG model supports genomic profiling of advanced GCT, particularly for patients in the IGCCCG intermediate- and poor-risk groups, to enhance risk

Fig 4. Progression-free survival (PFS) by *TP53/MDM2* status (wild type valtered) demonstrated superior PFS for patients with wild-type germ cell tumor.

stratification and facilitate clinical trials of novel treatment strategies.

Potentially actionable alterations were identified in 55% of patients with cisplatin-resistant disease (Appendix Table A2, online only). The most common of these was *MDM2* amplification, which has immediate therapeutic implications because seven potent inhibitors of *MDM2* are under study in clinical trials with promising results.¹⁵ Furthermore, sensitivity to *MDM2* inhibitors is more pronounced in neuroblastoma cell lines that harbor *MYCN* amplification, an alteration observed in four additional *TP53* wild-type but cisplatin-resistant tumors in our cohort.¹⁶ The promise of *MDM2* inhibitors in patients with GCT is also supported by in vitro studies of the *MDM2* inhibitor nutlin-3, which demonstrated strong antiproliferative and apoptotic activity and synergy with cisplatin in *TP53* wild-type, cisplatin-resistant GCT cell lines.^{17,18}

Potentially targetable alterations were also identified in the PI3K and RAS RTK pathways. Of particular note, nine activating mutations in *RAC1* were identified and functionally validated. *RAC1* is a Rho family GTPase that shares significant residue homology to *RAS* and plays a role in cell proliferation, motility, and drug resistance.¹³ *RAC1* mutations are rare in human cancers, with only 38 mutations identified in 6,076 samples (0.6%) subjected to WES by The Cancer Genome Atlas. Thus, to our knowledge, the 5% incidence of *RAC1* mutations in this cohort makes GCT the cancer type with the highest prevalence of *RAC1* mutations reported to date.¹⁴

There are several limitations of this study. Although we identified and validated a genetic basis for cisplatin resistance in a significant proportion of patients with cisplatin-resistant tumors, there remains a large subset of patients for whom cisplatin resistance cannot be explained by the sequencing techniques and methodologies used in this study. We are thus pursuing whole-genome, transcriptome, and epigenome analyses to further define the mechanisms of treatment resistance in such patients. Second, the rate of deaths in the cisplatin-resistant

cohort (23.1%) was lower than expected.¹ This probably reflects both the relatively short follow-up after progression to first-line chemotherapy for patients with cisplatin-resistant GCT in our cohort, as well as a high response rate to salvage treatment. In particular, high-dose chemotherapy with stem-cell transplantation has been demonstrated to overcome resistance to standard-dose cisplatin in a sizable proportion of patients with GCT progressing after first-line chemotherapy.^{19,20} There were too few recurrent genetic abnormalities to identify associations with response to specific salvage chemotherapy regimens in our cohort, but these associations should be explored in future studies. Third, 49 resistant samples were mixed GCTs that contained teratoma ($n = 49$), which is known to be cisplatin resistant. Although pure teratomas were excluded from our cohort and we strove to sequence only the nonteratomatous portion of these tumors, we did not perform microdissection. Therefore, a small amount of DNA from the teratoma component could have been sequenced in some cases. However, the finding of *TP53* and *MDM2* alterations within tumors that did not contain any teratoma makes it unlikely that this had any meaningful effect on our results. Finally, although we identified actionable genomic alterations within tumors from a significant proportion of patients with cisplatin resistance, the frequency of each specific alteration was low, posing a challenge to clinical trial enrollment and targeted drug development in this disease.

Despite these limitations, this series provides, to our knowledge, the first evidence for a genomic basis for cisplatin resistance among a significant proportion of patients with advanced GCT. Our data also provides a potential genomic explanation for the adverse prognosis associated with specific clinical features. In particular, we identified a high rate of *TP53* alterations among primary mediastinal nonseminomas, the group of GCT with the highest rates of chemoresistance and poorest outcomes. Furthermore, the prognostic significance of *TP53* pathway alterations independent of the current clinically based IGCCCG prognostic model indicates the potential for genomic sequencing to improve upon current risk stratification and provides a rationale for genomic profiling in patients with advanced GCT. Finally, the presence of actionable genomic alterations in nearly half of cisplatin-resistant GCT suggests novel treatment approaches may be of benefit for patients with refractory GCT and indicates a potential advantage of prospective genomic characterization of these tumors.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at www.jco.org.

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Genetic Determinants of Cisplatin Resistance in Patients With Advanced Germ Cell Tumors

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Appendix

METHODS

Whole-Exome Sequencing

Of 19 samples, 14 (74%) had $\geq 50\%$ tumor purity. Any sample with evidence of malignant transformation of teratoma was excluded. Matched normal DNA was obtained from peripheral blood in five patients, adjacent nontestis normal parenchyma (eg, lung) in four patients, and adjacent non-neoplastic testis tissue in 10 patients. When adjacent testis tissue was used for comparison against normal, the absence of intratubular germ cell neoplasia was pathologically confirmed. Primary and metastatic lesions were included in this analysis as were both pre- and postchemotherapy specimens. Between 1.5 and 2 μg of genomic DNA was captured by hybridization using the SureSelect XT Human All Exon V4 (Agilent Technologies, Santa Clara, CA). Libraries were prepared according to manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the libraries was performed, followed by sample barcoding, and all specimens were sequenced on a HiSeq2500 in 100 base pair (bp) paired-end runs using the TruSeq SBS Kit V5 (Illumina, San Diego, CA).

Sequence Alignment and Mutation Detection

Sequence alignment and mutation identification were performed as previously described (Johnson BE, et al: *Science* 343:189-193, 2014). Briefly, paired-end sequencing data from exome-capture libraries were aligned to the reference human genome (hg19) with the Burrows-Wheeler Aligner (Li H, et al: *Bioinformatics* 25:1754-1760, 2009). Deduplication, base quality recalibration, and multiple-sequence realignment were performed using Picard suite (<https://broadinstitute.github.io/picard/>) and the Genome Analysis Toolkit (<https://software.broadinstitute.org/gatk/>) before mutation detection (McKenna A, et al: *Genome Res* 20:1297-1303, 2010; and DePristo MA, et al: *Nat Genet* 43:491-498, 2011). BAM files were sorted and analyzed for both point mutations and small insertions and deletions (indels) of less than 50 bp. Single-nucleotide variants were detected using MuTect (<https://www.broadinstitute.org/cancer/cga/mutect>), a Bayesian framework for the detection of somatic mutations and indels using Pindel (<https://github.com/genome/pindel>; Cibulskis K, et al: *Nat Biotechnol* 31:213-219, 2013; and Ye K, et al: *Bioinformatics* 25:2865-2871, 2009).

RAC1 Experiments

The coding sequences for wild-type *RAC1*, *RAC1*^{G12R}, *RAC1*^{P29S}, *RAC1*^{P34R}, *RAC1*^{Q61K}, and *RAC1*^{Q61R} were obtained from Genewiz (South Plainfield, NJ), amplified by PCR to include an N-terminal 3 $\times$ FLAG epitope tag (Sigma-Aldrich, Saint Louis, Missouri), and subcloned into a pcDNA3 mammalian expression vector (Life Technologies, Grand Island, NY). Expression constructs were transfected into 293T cells using Lipofectamine 2000 (Life Technologies), and cells were collected and lysed in radioimmunoprecipitation assay buffer lysis buffer after 48 hours. GTP-bound Rac1, or active Rac1, was isolated via immunoprecipitation using recombinant p21-binding domain (PBD) of PAK1 (PAK1-PBD; Active Rac1 Detection Kit, catalog no. 8815; Cell Signaling Technology, Danvers, MA), according to the manufacturer's instructions. Immunoblots were performed using anti*RAC1* (antibody no. 4651), anti

MEK1/2

 (S217/221) (antibody no. 9121), anti

PAK1

 (T423; (antibody no. 2601), and anti

MEK1/2

 (antibody no. 9122) primary antibodies (all from Cell Signaling Technology). Secondary antibodies were then detected using a Fuji LAS-4000 imager (GE Healthcare Life Sciences, Pittsburgh, PA).

Table A1. Number of Total and Nonsynonymous Mutations in the Discovery Cohort (n = 19)

Sample	Cisplatin-Sensitivity	Mutations	
		Total	Nonsynonymous
1	Resistant	52	38
2	Resistant	208	137
3	Resistant	18	12
4	Resistant	96	65
5	Resistant	18	16
6	Resistant	117	83
7	Resistant	10	8
8	Resistant	75	51
9	Resistant	17	13
10	Resistant	39	33
11	Sensitive	19	13
12	Sensitive	31	24
13	Sensitive	10	8
14	Sensitive	21	16
15	Sensitive	15	7
16	Sensitive	37	28
17	Sensitive	24	17
18	Sensitive	22	14
19	Sensitive	14	10

Table A2. Potentially Actionable Alterations (n = 75) Among 104 Cisplatin-Resistant Germ Cell Tumors

Pathway	Gene	Alteration/Mutation	Potentially Effective Drug(s)	Citation (PMID)
TP53	<i>MDM2</i>	Amplification	Nutlin-3	19560254
TP53	<i>MDM2</i>	Amplification	Nutlin-3	19560254
TP53	<i>MDM2</i>	Amplification	Nutlin-3	19560254
TP53	<i>MDM2</i>	Amplification	Nutlin-3	19560254
TP53	<i>MDM2</i>	Amplification	Nutlin-3	19560254
TP53	<i>MDM2</i>	Amplification	Nutlin-3	19560254
TP53	<i>MDM2</i>	Amplification	Nutlin-3	19560254
TP53	<i>MYCN</i>	Amplification	Nutlin-3	21460101
TP53	<i>MYCN</i>	Amplification	Nutlin-3	21460101
TP53	<i>MYCN</i>	Amplification	Nutlin-3	21460101
TP53	<i>MYCN</i>	Amplification	Nutlin-3	21460101
TP53	<i>MYCN</i>	Amplification	Nutlin-3	21460101
TP53	<i>MAX</i>	R60Q	Nutlin-3	26619011
RTK	<i>KIT</i>	W557G	Imatinib, sunitinib	14645423, 22261812
RTK	<i>KIT</i>	D816V	Midostaurin (TKI)	21641642
RTK	<i>KIT</i>	W557S	Imatinib (TKI)	14645423
RTK	<i>KIT</i>	D419N	Imatinib	16081693
RTK	<i>KIT</i>	D820Y	Nilotinib (TKI)	17699867
RTK	<i>MET</i>	Amplification	MET inhibitor	16461907
RTK	<i>KDR</i>	Amplification	VEGF inhibitors	22130231
RTK	<i>FOXO1</i>	Deletion	Trifluoperazine hydrochloride	22653055
RTK	<i>CBL X410spl</i>	Splice variant	KIT/SRC TKI	23832011
RTK	<i>FGFR3-TACC3</i>	Fusion	FGFR3 inhibitor	21119661
RAS	<i>KRAS</i>	A146P	MEK inhibitor (PD0325901)	18952427
RAS	<i>KRAS</i>	A146P	MEK inhibitor (PD0325901)	18952427
RAS	<i>KRAS</i>	A146V	MEK inhibitor (PD0325901)	18952427
RAS	<i>KRAS</i>	G12D/V	MEK 1/2 inhibitor	21118963
RAS	<i>KRAS</i>	G12R	MEK 1/2 inhibitor	21118963
RAS	<i>KRAS</i>	G12V	MEK 1/2 inhibitor	21118963
RAS	<i>KRAS</i>	G12A	MEK 1/2 inhibitor	21118963
RAS	<i>KRAS</i>	G12A	MEK 1/2 inhibitor	21118963
RAS	<i>KRAS</i>	G12D	MEK 1/2 inhibitor	21118963
RAS	<i>KRAS</i>	G12D	MEK 1/2 inhibitor	21118963

(continued on following page)

Genetic Determinants of Cisplatin Resistance in Advanced GCT

Table A2. Potentially Actionable Alterations (n = 75) Among 104 Cisplatin-Resistant Germ Cell Tumors (continued)

Pathway	Gene	Alteration/Mutation	Potentially Effective Drug(s)	Citation (PMID)
RAS	<i>KRAS</i>	G12S	MEK 1/2 inhibitor	21118963
RAS	<i>KRAS</i>	G60S	MEK 1/2 inhibitor	26619011
RAS	<i>KRAS</i>	G60V	MEK 1/2 inhibitor	26619011
RAS	<i>KRAS</i>	K117R	MEK1/2 inhibitor	22205714
RAS	<i>NRAS</i>	Q61H	MEK1/2 inhibitors (binimetinib)	23414587
RAS	<i>NRAS</i>	Q61P	MEK1/2 inhibitors (binimetinib)	23414587
RAS	<i>NRAS</i>	G12D	MEK1/2 inhibitors (binimetinib)	23414587
RAS	<i>BRAF</i>	D594G	Sorafenib	20141835
RAS	<i>BRAF</i>	D594N	Sorafenib	20141835
RAS	<i>BRAF</i>	G466E	Dasatinib	22649091
RAS	<i>RAC1</i>	G12R	RAC1 inhibitor (NSC23766)	19509242
RAS	<i>RAC1</i>	G12V	RAC1 inhibitor (NSC23766)	19509242
RAS	<i>RAC1</i>	Q61K	RAC1 inhibitor (NSC23766)	19509242
RAS	<i>RAC1</i>	Q61K	RAC1 inhibitor (NSC23766)	19509242
RAS / G protein activation	<i>GNAQ</i>	Q209P	MEK1/2 inhibitors (trametinib, selumetinib)	17332304, 23846731
PI3K/MTOR	<i>AKT1</i>	Amplification	AKT1/mTOR/PI3K inhibition	25498304
PI3K/MTOR	<i>PIK3CA</i>	E542K	PI3K inhibitors	20453058
PI3K/MTOR	<i>PIK3CA</i>	E542K	PI3K inhibitors	20453058
PI3K/MTOR	<i>PIK3CA</i>	E542K	PI3K inhibitors	20453058
PI3K/MTOR	<i>PTEN</i>	L70spl	PI3K inhibitors	25088603
PI3K/MTOR	<i>PTEN</i>	P213fs	PI3K inhibitors	25088603
PI3K/MTOR	<i>PTEN</i>	V317fs	PI3K inhibitors	25088603
PI3K/MTOR	<i>PTEN</i>	Deletion	PI3K inhibitors	25088603
PI3K/MTOR	<i>PTEN</i>	Deletion	PI3K inhibitors	25088603
PI3K/MTOR	<i>TSC1</i>	Deletion	Everolimus	22923433
PI3K/MTOR	<i>TSC2</i>	A889G	Everolimus	22903760
Tumor suppressors	<i>SETD2</i>	T387fs	PI3K/AKT pathway inhibitor (TGX221)	25853938
Tumor suppressors	<i>APC</i>	S1847*	(APC: G007-LK)	23539443
Tumor suppressors	<i>APC</i>	S457*	Tankyrase inhibitor	23539443
Tumor suppressors	<i>BRCA2</i>	Deletion	PARP inhibitors	25750544
Tumor suppressors	<i>PTPRD</i>	R427*	Stat3 inhibitor (JSI-124)	26267899
WNT	<i>AXIN1</i>	W564*	Wnt targeted agents	10700176
WNT	<i>FAT1</i>	Deletion	Wnt targeted agents	15735684
WNT	<i>FAT1</i>	E2874fs	Wnt targeted agents	15735684
WNT	<i>FAT1</i>	V1577fs	Wnt targeted agents	15735684
Apoptosis	<i>MCL1</i>	Amplification	HSP90 inhibitor	22516262
Apoptosis	<i>MCL1</i>	Amplification	HSP90 inhibitor	22516262
Cell cycle	<i>CCND3</i>	Amplification	PD-0332991 (palbociclib)	23079656
Cell cycle	<i>CCND3</i>	Amplification	PD-0332991 (palbociclib)	23079656
Chromatin modifier	<i>EP300</i>	C351*	HDAC inhibitor	24732587
Chromatin modifier	<i>CREBBP</i>	R1498*	HDAC inhibitor	21390126
IGF	<i>IRS1</i>	Amplification	NT157 (IRS1/TKI)	25267499

Abbreviations: fs, frameshift; IGF, insulin-like growth factor; PMID, PubMed identification number; RTK, receptor tyrosine kinase; spl, splice variant; TKI, tyrosine kinase inhibitor.

*Each row represents a separate alteration. Samples from some patients had more than one alteration.