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Germ cell tumors and associated hematologic malignancies evolve from a common shared precursor

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ABSTRACT
Germ cell tumors (GCTs) are the most common cancer in men between the ages of 15-40. While most patients are cured, those with disease arising in the mediastinum have distinctly poor outcomes. One in every 17 patients with primary mediastinal non-seminomatous GCTs develop an incurable hematologic malignancy and prior data intriguingly suggests a clonal relationship exists between hematologic malignancies and GCTs in these cases. To date however, the precise clonal relationship between GCTs and the diverse additional somatic malignancies arising in such individuals has not been determined. Here, we traced the clonal evolution and characterized the genetic features of each neoplasm from a cohort of fifteen patients with GCTs and associated hematologic malignancies. We discovered that GCTs and hematologic malignancies developing in such individuals evolved from a common shared precursor, nearly all of which harbored allelically imbalanced TP53 and/or RAS pathway mutations. Hematologic malignancies arising in this setting genetically resembled mediastinal GCTs rather than de novo myeloid neoplasms. Our findings argue that this scenario represents a unique clinical syndrome, distinct from de novo GCTs or hematologic malignancies, initiated by an ancestral precursor which gives rise to the parallel evolution of GCTs and blood cancers in these patients.
INTRODUCTION

Germ cell tumors (GCTs) are a model of curable cancer as most patients with metastatic GCTs are successfully treated with cisplatin-based chemotherapy (1). However, up to 30% of patients with advanced GCTs develop cisplatin resistant disease, which requires intensive salvage treatment and has a 50% risk of cancer-related death (2). In particular, primary mediastinal non-seminomatous germ cell tumors (PM NSGCTs) have an adverse prognosis and a high frequency of cisplatin resistance (3). Moreover, diverse forms of somatic type malignancies including sarcomas, carcinomas, and a variety of hematologic malignancies can arise in patients with mediastinal GCTs (4, 5). For example, one in every 17 patients with PM NSGCT will develop a hematologic malignancy (5, 6), a clinical presentation associated with a dismal median survival of less than six months.

Although initially presumed to represent a therapy-induced secondary complication of chemotherapy, a shared clonal origin for GCTs and hematologic malignancies arising in the same individual has been suggested by the occasional shared presence of isochromosome 12p (i(12p)), a clonal marker common in GCTs but absent in hematologic cancers (7-10). While the identification of i(12p) in hematologic malignancies arising in GCT patients suggests a clonal relationship between the two disorders (7-10), the evolutionary dynamics and comprehensive characterization of the genomic features of this syndrome have thus far been lacking. Given the totipotent nature of GCTs, it has been postulated that hematologic malignancies arising in this setting are derived directly from cells within the fully formed GCT that have the capacity to differentiate into hematopoietic tissue (11, 12). However, a competing hypothesis is that a progenitor with the capacity to differentiate into germ cell and hematopoietic lineages harbors the initiating genetic alterations and drives development of both malignancies (10) (Supplemental Figure 1). To trace the clonal origins of these multiple malignancies within individual patients and understand their comprehensive genomic features, we performed broad mutational and copy number analyses of GCTs, the hematologic malignancies developing in these individuals, and patient-matched normal DNA from a cohort of fifteen patients.
RESULTS

We identified 15 individuals diagnosed with GCT and co-incident hematologic malignancies over a 21-year period (1998-2019; Figure 1A; Supplemental Table 1; Methods). Despite allowing the collection of GCTs from any gender and histologic subtype, all patients developing GCTs and concurrent blood cancers were males between the age of 18-33 (median age 25) with PM NSGCTs. The most common GCT histology was a teratoma component (present in 73% and as the predominant histology in 60% of cases) mixed with non-seminomatous components, most commonly a yolk sac tumor component (present in 53%; more details in Supplemental Table 1).

Overall, the median time between GCT and blood cancer diagnoses was only 4.8 months, while four patients concurrently developed their GCT and hematologic cancer prior to any anti-cancer therapy (Figure 1A). The most common hematologic malignancy was acute myeloid leukemia (AML) in 53% of patients, but myelodysplastic syndromes (MDS), histiocytic sarcoma, and chronic myelomonocytic leukemia (CMML) were also diagnosed in two or more patients each. Nearly half of the AMLs seen in this setting were acute megakaryoblastic leukemias (M7 AML). However, one-third of the cohort developed multiple distinct clinical subtypes of hematologic malignancies, often in a temporally distinct manner (Figure 1A; Supplemental Table 1).

Consistent with prior reports documenting the poor survival of patients who develop both GCTs and hematologic malignancies (7-10), the median survival of this cohort was 6.3 months (95% confidence interval (CI) 4.6 - 25.2 months) (Figure 1B). Only one patient from the cohort is alive to date (and the cause of death for each patient is noted in Supplemental Table 1). In order to understand the genetic features of the GCTs and hematologic malignancies arising in this aggressive clinical setting, in 11 patients with available tissue, we performed targeted and/or whole-exome sequencing (WES) of tumor DNA from the GCT, hematologic malignancy (blood, marrow, or biopsy of affected solid tissue), and patient-matched normal controls using DNA from fingernails. The genetic features of each tumor in these patients were then compared to those of a cohort of 51 patients with mediastinal GCTs (including 10 with seminoma and 41 with non-seminoma histology) who did not develop additional malignancies after long-term clinical follow-up as well as 200 de novo AML patients of a similar age range (from the AML TCGA dataset (13); (Figure 1C-D, Supplemental Figure 2)).

Blood cancers developing in the setting of PM NSGCTs were far more genetically similar to PM NSGCTs overall than to de novo AML. For example, i(12p) was commonly seen in mediastinal GCTs and hematologic malignancies in our cohort, whereas i(12p) was never
observed in de novo AML (Figure 1C-D, Supplemental Table 2). Although a complex karyotype with multiple chromosome abnormalities was evident in the majority of hematologic malignancies developing in the setting of GCTs, i(12p) could be identified as an isolated clone by conventional cytogenetics in several of these individuals, suggesting i(12p) as an early event in these cases (Supplemental Figure 3, Supplemental Table 3). In addition to i(12p), shared mutations in TP53 were present in 91% of patients with mediastinal GCTs with hematologic malignancies versus 61% of patients with mediastinal GCTs lacking hematologic malignancies and in only 9% of patients with de novo AML (Figure 1C). Given recent data on the association of TP53 pathway alterations and cisplatin resistance in GCTs (14) and the known adverse prognostic effect of TP53 alterations in myeloid neoplasms (15-17), the nearly obligatory presence of TP53 mutations in GCTs and their associated secondary blood cancers provides one basis for the short survival of these patients. Activating mutations in K/NRAS accounted for 63% and 37% of mediastinal GCTs with hematologic malignancies and without accompanying hematologic malignancies, respectively; but were present in only 13% of de novo AML. In contrast to shared genetic similarities in i(12p), TP53 alterations, and mutations activating RAS/PI3K signaling in GCTs and their associated blood cancers, blood cancers arising in association with GCTs lacked mutations in the most commonly mutated genes in AML, MDS, or clonal hematopoiesis (such as FLT3, DNMT3A, TET2, and NPM1 mutations; Figure 1C-D).

These findings suggest that the hematologic malignancies developing in the setting of GCTs are clonally related to mediastinal GCTs and have a pathogenesis distinct from other hematopoietic malignancies. This represents a possible basis for their treatment refractoriness, as these blood cancers are molecularly distinct from the hematologic malignancies they histologically mimic.

We next sought to trace the evolutionary origin of each distinct cancer type developing in these patients by utilizing the somatic mutational and DNA copy number data from WES in 5 patients (Figures 2, 3C, and 4F). In every such patient, we identified divergent genetic evolution of the GCT and blood cancer, with putative driver alterations unique to each malignancy (sometimes in the same gene). In contrast to prior data that secondary malignancies developing in the setting of GCTs represent malignant transformation of differentiated GCT components such as teratoma and yolk sac tumor (11, 12), the present data suggest instead that these malignancies evolved independently from a shared ancestral clone common to both malignancies (Supplemental Figure 1). For example, in one 24-year old male with concurrent PM NSGCT and CMML, both cancers had i(12p) and KRAS G12S mutations, along with eight additional shared truncal mutations of unknown functional significance (Figure 2A-C). These alterations were then followed by the independent acquisition of a distinct set of
mutations (including oncogenic mutations) and copy number alterations (CNAs) arising after a branching of their disease evolution, thereby indicating that both conditions developed from a shared precursor (as opposed to dedifferentiation or histologic transformation of mature elements in the GCT into the subsequent hematologic malignancy as has been speculated).

In nearly every case, the common precursor for the GCT and blood cancer harbored either a TP53 or RAS pathway alteration (Figure 2D-J). For example, we identified one 28-year old male with a PM NSGCT and a diagnosis of high-risk MDS wherein both malignancies developed from a shared KRAS G12D-mutant precursor bearing identical CNAs on chromosomes 13 and 17 (Figure 2G-J). Each malignancy thereafter acquired distinct somatic mutations and CNAs including TP53 hotspot mutations, each of which underwent independent copy-neutral loss-of-heterozygosity (CN LOH), thus evolving from a shared common ancestor followed by divergent parallel evolution of each disease (Figure 2J).

Even in cases where patients harbored multiple temporally distinct blood cancers, each subtype of hematologic malignancy was found to have its own unique precursor shared with the GCT. For example, we studied a 19-year old patient who developed successive diagnoses of histiocytic sarcoma, CMML, and AML within 18 months of GCT diagnosis (Figure 3). Tracing these four individual cancers via their somatic mutational profiles revealed that all were clonally related and derived from a common precursor bearing a TP53 Q144Rfs*28 mutation with CN LOH, a PI3KCD mutation, and shared CNAs on chromosomes 1, 2, and 5. From this shared precursor, the GCT and AML evolved from a daughter precursor bearing 1q and 21q gain while the histiocytic sarcoma and CMML evolved from a separate daughter precursor. Further divergence between the CMML and histiocytic sarcoma is highlighted by a unique NRAS Q61H mutation in the histiocytic sarcoma (Figure 3B-C). Interestingly, the inferred clonal relatedness of the individual malignancies did not match the order of diagnosis, supporting the hypothesis that all malignancies were derived from a common precursor clone, rather than serially transformed from GCT elements in the mediastinal tumor. Notably, this patient’s GCT did not have the i12p abnormality and thus might not have been identified as having clonally related GCT and leukemia based on karyotype alone.

High dose chemotherapy and autologous stem cell transplantation (auto-SCT) is a treatment for relapsed GCTs (18), a clinical scenario encompassing some of the GCT patients in this study. Given recent recognition for the potential for contamination of hematopoietic stem cell transplant products with clonal hematopoiesis (CH) (19), we next sought to determine if there was CH in the G-CSF-mobilized stem cell products collected from individuals with high risk PM GCTs. We therefore performed genetic analysis of G-CSF-mobilized hematopoietic
stem cell products from five primary mediastinal GCT patients who had undergone stem collection for potential autologous transplantation (Supplemental Table 3). Only one patient from this cohort developed a subsequent hematologic malignancy, despite undergoing high-dose chemotherapy with auto-SCT as treatment for their high-risk GCT, he developed a clonally related MDS shortly thereafter (one year after GCT diagnosis) (Figure 4A). After a subsequent allogeneic SCT, the patient developed a second clinically and histologically distinct hematologic malignancy- a histiocytic sarcoma (Figure 4B). WES revealed that all three tumors were host-derived and shared a somatic BCOR G943D mutation as well as an RRAS2 activating mutation with CN LOH (Figure 4C-F). Every disease thereafter acquired TP53 mutations with CN LOH (Figure 4D-G and Supplemental Figure 4). Again, the acquisition of a different TP53 mutation and successive independent LOH event in the histiocytic sarcoma suggest secondary malignancies developing from a precursor clone that also gave rise to the GCT (Figure 4D-G). To confirm, we sequenced the auto-stem cell product, which was collected from the patient prior to being diagnosed with hematologic malignancies, only to find that the mobilized stem cell product did not contain any mutations (Figure 4C). As with this index case, none of four additional PM GCT patients with auto-SCT products sequenced had evidence of CH (Supplemental Table 3), which is consistent with the rarity of CH in GCT patients overall (20). Together, these data suggest that the hematologic malignancies seen in these patients did not arise from the hematopoietic system, but rather developed from the same cell-of-origin as the GCT.
DISCUSSION
The development of clonally related somatic type cancers in the setting of GCTs has been recognized for nearly 30 years (5) and is marked by a very poor outcome, despite modern therapy for both cancer types. To date, the challenge of capturing both sets of malignancies in this aggressive clinical scenario combined with its relative rarity have limited efforts to understand this clinical phenomenon. Here we identify the precise clonal relationships of the tumors arising in these patients, the genetic features of primary mediastinal GCTs in general, as well as the subset which develop secondary hematologic malignancies. These first comprehensive maps of the somatic mutations and CNAs across solid and liquid malignancies in such individuals identify a common shared precursor in the germ cell lineage from which both the GCT and other malignancies develop. As such, the hematologic malignancies developing in these patients are genetically more similar to primary mediastinal GCTs than to de novo MDS or AML and lack many of the hallmark genomic drivers of myeloid malignancies. At the same time, the fact that hematologic malignancies bearing i(12p) do not develop unless accompanied by a GCT strongly suggests that hematologic malignancies present in this setting derive from a precursor along the GCT lineage.

All patients here who developed a hematologic malignancy clonally related to GCT had a primary mediastinal non-seminomatous GCT (PM NSGCT) and we did not identify any patients with testicular or seminomatous GCTs that developed a clonally related hematologic malignancy. While this is consistent with prior reports (7-10), the basis for the specific association between somatic type hematologic malignancies and PM NSGCTs remains unclear. Overall, our data are consistent with the model that PM NSGCTs are derived from transformed primordial germ cells lodged in the mediastinum, which have been reprogrammed to embryonal carcinoma cells and are believed to be the initiating cells of NSGCTs (21). Indeed, none of the hematologic malignancies here were preceded by a pure seminoma, making it unlikely that they can be derived directly from a primordial germ cell. Thus, we hypothesize that the precursor to both PM NSGCTs and clonally related hematologic malignancies in these individuals may be embryonal carcinoma cells, which have the potential to differentiate towards all components of a NSGCT as well as hematopoietic precursor cells. This is distinct from prior studies (11, 12) which postulated that hematologic malignancies in these cases develop from teratomas or yolk sac elements within the GCT.

The unique genetic features of hematologic malignancies in GCT patients along with the high frequency of TP53 alterations likely explains the frequent cisplatin resistance in this patient population as well the ineffectiveness of therapies utilized for high-risk MDS and AML. This
clinical scenario is particularly unique given the co-occurrence of multiple, otherwise unrelated rare cancer types in a young adult population and the fact that this condition is invariably fatal. As such, future studies to evaluate for potential germline genetic predisposition for the development of hematologic malignancies in the setting of GCTs are warranted. Of note, two patients with PM NSGCT that developed hematologic malignancies here had clinical diagnoses of Klinefelter syndrome, consistent with prior data regarding increased incidence of PM NSGCT in patients with Klinefelter syndrome (22). Although none of the 41 additional PM NSGCT patients without hematologic malignancies had known Klinefelter syndrome, further germline genomic analysis would be needed to evaluate for potential mosaicism or subclinical Klinefelter syndrome in these individuals. Of note, we identify far more diverse forms of myeloid neoplasms developing in this setting than appreciated in prior studies and observed that multiple genetically distinct myeloid neoplasms often develop in individual patients affected by this condition. Overall, these findings argue that the development of blood cancers in patients with GCTs represents a unique clinical syndrome wherein the aggressive hematologic malignancies in these individuals do not arise from the hematopoietic system, like de novo hematologic malignancies, but rather develop from the same cell-of-origin as the germ cell tumor.
METHODS

Pathology review
All samples underwent detailed pathology review to confirm diagnosis by both specialized hematopathologists (C.H., K.P.-D.) as well genitourinary pathologists (H.A.A.) and cytogenetics of both GCT and hematologic malignancies were reviewed by dedicated cytogeneticists (Y.Z., U.A.).

Genomic sequencing and mutation calling
Next-generation sequencing (NGS) was performed on DNA extracted from tumor and matched normal DNA samples from fingernails. Patient samples were sequenced using the MSK-IMPACT targeted sequencing panel, with somatic mutations (substitutions and small insertions and deletions), gene-level focal copy number alterations, and structural rearrangements detected with a clinically validated pipeline as previously described (23, 24). Whole exome sequencing and mutational analysis was performed on a subset of cases as previously described (25). Depth coverage at the positions of non-truncal mutations in the tumors in which they were not detected was calculated, median 196 (IQR: 112-312). Those loci with tumor depth < 20 (the same depth cut off used to filter called mutations) were excluded from all related samples. The OncoKB knowledgebase was used to identify oncogenic alterations (26). For the post allogenic transplant histiocytic sarcoma sample from patient two (Figure 4), likely true somatic mutations were distinguished from donor derived contamination using the VAF of mutations previously identified in pre-allogenic samples from the same patient along with the known zygosity of those mutations in said samples (Supplemental Figure 4). This sample was also excluded from copy number analysis. Patient three’s NRAS mutation in their histiocytic sarcoma sample was initially identified in additional IMPACT sequencing, read support 2.05% (14/683), reaching the threshold for reporting oncogenic/hotspot mutations. Read support in the WES was 1.02% (2/197). The same NRAS mutation was not present in other the histologic components via IMPACT sequencing (GCT: 0/874; CMML: 1/358; AML: 0/574).

Allele-specific copy number analysis and cancer cell fractions
Genome-wide total and allele-specific DNA copy number, purity, and ploidy were calculated for all exome data (FACETS version 0.5.6 (27)). Only clonal copy number alterations were considered, i.e. copy number segments with a cellular fraction ≥ 80% of estimated purity. The expected number of copies for each mutation was generated based on observed VAF and local ploidy (28). Cancer cell fractions were calculated using a binomial distribution and maximum likelihood estimation normalized to produce posterior probabilities (29).

Phylogenetic reconstruction
Clone trees were manually constructed based on the most parsimonious sequence of events using mutations and copy number events. Branch lengths represented as the number of shared or unique alterations.

Data availability
Whole exome data are deposited at dbGap under accession ID phs2231.V1 and IMPACT mutation calls are deposited via the cBioPortal for Cancer Genomics https://www.cbioportal.org/study/summary?id=gct_msk_2020).

Statistics
Progression-free survival (PFS) 95% confidence intervals were calculated using the R survival package (v.3.1-8).
**Study approval**
Studies were approved by the Institutional Review Board of Memorial Sloan Kettering Cancer Center and conducted in accordance to the Declaration of Helsinki protocol (IRB protocols 15-021, 06-107). All patients provided written informed consent before solid tumor tissue, fingernails, or peripheral blood or bone marrow mononuclear cells were utilized.
Author contributions

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Figure 1. Genetic and clinical characteristics of patients with germ cell tumors (GCTs) and concomitant hematologic malignancies. (A) Timeline of diagnoses and histologic characteristics of GCT and blood cancers from all 15 patients. Each patient is shown in a row on the x-axis and timeline of diagnosis is shown in the y-axis. Abbreviations: AML: acute myeloid leukemia; CMML: chronic myelomonocytic leukemia; HLH: hemophagocytic lymphohistiocytosis; MDS: myelodysplastic syndrome. (B) Kaplan-Meier curve of the patients from (A). Survival is shown as time from diagnosis of germ cell tumor. Median survival is 6.3 months (95% CI 4.6 - 25.2 months). (C) Shown are the most prevalent genetic alterations in patients with mediastinal GCTs and no secondary malignancy diagnosis (left; n=51),
mediastinal GCT with hematologic malignancy (middle; n=11; composite for GCT and hematologic malignancy samples shown), or de novo AML (n=200, from the AML TCGA (13)). (D) DNA copy number alterations (CNAs) in the same cohorts of patients from (C) (genomic gains and losses are red and blue, respectively). GCT and hematologic malignancy samples displayed separately for CNAs (GCT=8, HM=9 patients (11 samples)).
Figure 2. Genetic evolution of germ cell tumors (GCTs) and hematologic malignancies from a common ancestral clone. Whole exome sequencing was performed in 5 patients, 3 shown here and 2 shown in Figures 3-4. (A) Shown are the somatic mutations (known oncogenic and variants of undetermined significance (VUS)) and their fraction of tumor cells mutated (cancer cell fraction, CCF), the (B) allelic DNA copy number, and (C) inferred evolutionary relationships of a GCT and associated myelodysplastic syndrome (MDS) in a 24-year old male with both diagnoses. An ancestral clone with shared defined by isochromosome 12p and a KRAS G12S mutation (amongst other shared mutations) was detected followed by independent acquisition of numerous distinct mutations. (D-F) Similar CCF (D), allele copy number (E), and evolutionary relationships (F) in a 28-year old with shared GCT and AML. In this case, both tumors derived from a TP53 R248L-mutant ancestral precursor which underwent copy neutral loss-of-heterozygosity (CN LOH). (G-J) In another patient, a KRAS G12D mutant precursor gave rise to GCT and MDS, each of which later independently acquired TP53 hotspot mutations with CN LOH. (I) Aligned TP53 reads over the regions of mutations, highlighting
mutual exclusivity of each mutation across the GCT and MDS samples. Abbreviations: WES: whole exome sequencing; GCT: germ cell tumor; AML: acute myeloid leukemia; CMML: chronic myelomonocytic leukemia; MDS: myelodysplastic syndrome.
Figure 3. Genomic evolution of multiple clinically and genetically distinct hematologic malignancies arising in the setting of a clonally related germ cell tumor (GCT). (A) Cancer cell fraction (CCF) of mutations, (B) allele copy number (middle), (C) evolutionary relationship, and (D) histopathology (hematoxylin and eosin stain) of a 19-year old patient successively diagnosed with a GCT, histiocytic sarcoma, chronic myelomonocytic leukemia (CMML), and acute myeloid leukemia (AML). Black scale bars: 200 micrometers; white scale bars: 1 micrometer. The timeline of acquisition of each diagnosis is shown at the top of (A). In this case, TP53 Q144Rfs*26 mutation undergoing copy neutral loss-of-heterozygosity defined the ancestral precursor with a PIK3CD mutation that gave rise to all four malignancies. This was followed by divergent evolution of one intermediate precursor which gave rise to the GCT and AML and another which resulted in development of the histiocytic sarcoma and CMML.
Abbreviations: GCT: germ cell tumor; AML: acute myeloid leukemia; CMML: chronic myelomonocytic leukemia; MDS: myelodysplastic syndrome.
Figure 4. Lack of clonal hematopoiesis in autologous stem cell products from germ cell tumor (GCT) patients developing hematologic malignancies. (A) Clinical course of a 25-year old patient with metastatic primary mediastinal non-seminomatous GCT who was treated with multiple salvage chemotherapy regimens (VIP: etoposide, ifosfamide, cisplatin; TICE: paclitaxel, ifosfamide, followed by high-dose carboplatin plus etoposide) as well as autologous stem cell transplant (SCT). This was followed by development of a myelodysplastic syndrome (MDS) one year after GCT diagnosis, treated by allogeneic SCT. A histiocytic sarcoma was then found one year after allogeneic SCT based on a PET/CT scan. (B) Histopathology (hematoxylin and eosin stain) of the yolk sac and teratoma component of the GCT as well as MDS and histiocytic sarcoma. Black scale bars: 200 micrometers; white scale bars: 1 micrometer. (C) Somatic mutations in TP53, BCOR, and NRAS illustrate the clonal relationships of the GCT, MDS, and histiocytic sarcoma versus the auto-SCT product. (D) The cancer cell fraction (CCF) of both shared and distinct somatic mutations in the GCT and MDS. (E) Copy number analysis of the GCT and MDS. (F) Evolutionary relationship of the GCT, MDS, and histiocytic sarcoma. (G) Aligned TP53 reads over the regions of mutations, highlighting mutual exclusivity of each mutation across the GCT and MDS samples compared to the histiocytic sarcoma sample. Abbreviations: GCT: germ cell tumor; MDS: myelodysplastic syndrome.