Original Article

Intratumoral Heterogeneity: Role of Differentiation in a Potentially Lethal Phenotype of Testicular Cancer

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BACKGROUND: Intratumoral heterogeneity presents a major obstacle to the widespread implementation of precision medicine. The authors assessed the origin of intratumoral heterogeneity in nonseminomatous germ cell tumor of the testis (NSGCT) and identified distinct tumor subtypes and a potentially lethal phenotype. **METHODS:** In this retrospective study, all consecutive patients who had been diagnosed with an NSGCT between January 2000 and December 2010 were evaluated. The histologic makeup of primary tumors and the clinical course of disease were determined for each patient. A Fine and Gray proportional hazards regression analysis was used to determine the prognostic risk factors, and the Gray test was used to detect differences in the cumulative incidence of cancer death. In a separate prospective study, next-generation sequencing was performed on tumor samples from 9 patients to identify any actionable mutations. **RESULTS:** Six hundred fifteen patients were included in this study. Multivariate analysis revealed that the presence of yolk sac tumor in the primary tumor ($P = .0003$) was associated with an unfavorable prognosis. NSGCT could be divided into 5 subgroups. Patients in the yolk sac-seminoma subgroup had the poorest clinical outcome ($P = .0015$). These tumors tended to undergo somatic transformation ($P < .0001$). Among the 9 NSGCTs that had a yolk sac tumor phenotype, no consistent gene mutation was detected. **CONCLUSIONS:** The current data suggest that intratumoral heterogeneity is caused in part by differentiation of pluripotent progenitor cells. Integrated or multimodal therapy may be effective at addressing intratumoral heterogeneity and treating distinct subtypes as well as a potentially lethal phenotype of NSGCT. ***Cancer* 2016;122:1836-43.** © 2016 The Authors. *Cancer* published by Wiley Periodicals, Inc. on behalf of *American Cancer Society*. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

KEYWORDS: integrated therapy, intratumoral heterogeneity, lethal phenotype, precision medicine, testicular cancer, yolk sac tumor.

INTRODUCTION

Intratumoral heterogeneity is prevalent in cancer. It poses a significant challenge to the basic premise and presents a major obstacle to the widespread implementation of precision medicine. One way to resolve the dilemma of intratumoral heterogeneity is to identify distinct tumor subtypes with unique cellular origins and molecular profiles.

Although the clinical relevance of intratumoral heterogeneity seems self-evident, its clinical validity remains unsolved. Currently, it is unclear whether intratumoral heterogeneity is derived from differentiation of aberrant progenitor cells or from mutation of driver genes. By elucidating the origin of intratumoral heterogeneity in a pertinent clinical model, our objective is to effectively translate intratumoral heterogeneity from a critical clinical observation to a practical clinical utility.

Nonseminomatous germ cell tumor of the testis (NSGCT) is a prototype cancer with intratumoral heterogeneity.[1] It is noteworthy that it is a curable solid tumor. Thus, lessons learned about NSGCT are invaluable in our effort to

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understand and cure other solid tumors. Because the histologic makeup of NSGCT is easily identifiable, its differentiation pattern can be readily deduced.[2] Hence, early germ cells may express embryonic and extraembryonic features. The embryonic components may form embryonal carcinoma and teratoma, whereas the extraembryonic components may form yolk sac tumor and choriocarcinoma. Yolk sac tumor contains endodermal and mesodermal elements. Choriocarcinoma is derived from the chorion. In contrast, seminomas originate from true gonadal cells.

If the evolution of cancer cells mimics the development of progenitor cells, then NSGCTs are model cancer stem cells and germ cells are ideal stem cells for the study of intratumoral heterogeneity.[3,4] Aberrant cells in the early stem-cell hierarchy are inherently pluripotent; they have the capacity to differentiate and form diverse histologic phenotypes (eg, embryonal carcinoma, choriocarcinoma, yolk sac tumor, seminoma, and teratoma). In contrast, defective cells in the same late stem-cell hierarchy give rise to tumors that are more homogeneous (ie, pure seminoma). It is worth noting that earlier phenotypes may include later ones (ie, mixed NSGCTs may contain a seminoma component), but not vice versa.

We propose that NSGCT is a useful clinical model in which elucidate the origin of intratumoral heterogeneity. Because orchiectomy and metastasectomy are routinely performed for NSGCTs, abundant tumor tissues are available for histologic and molecular analyses. A specific chromosome change, isochromosome 12p, is observed in 86% of germ cell tumors and all of their histologic components.[5] Similarly, it has been demonstrated that the molecular profiles of the various histologic components, primary and metastatic tumors, stromal and epithelial compartments, and teratomatous and somatically transformed constituents are highly concordant.[6-9] Because the various histologic components of NSGCT have a similar genetic signature, the discovery of a specific driver mutation in a particular NSGCT subtype seems both rational and feasible.

In this study, we investigated whether intratumoral heterogeneity could be used to identify distinct NSGCT subtypes with unique clinical features and biologic characteristics. We hypothesized that the identification of tumor subtypes would reveal a cellular or genetic origin of intratumoral heterogeneity. The discovery of actionable cellular or genetic targets for the purpose of precision medicine may lead to improved integrated or multimodal therapy for NSGCT in particular and for solid tumors in general.

MATERIALS AND METHODS

Patients

The Tumor Registry database at The University of Texas MD Anderson Cancer Center (Houston, Tex) (MD Anderson) was used to identify all consecutive patients who testicular cancer diagnosed from January 2000 to December 2010. Only patients who had nonseminomatous germ cell tumors were included, and patients who had pure seminoma were excluded. Other exclusion criteria included orchiectomy after chemotherapy, pathologic sample not available for review, nongerm cell tumor (ie, paratesticular tumor), age < 3 years with a pure yolk sac tumor or teratoma, and extragonadal germ cell tumor.

We evaluated the pathologic findings from all specimens of postchemotherapy retroperitoneal lymph node dissection (RPLND) and other surgeries. Specifically, we determined whether patients who had certain histologic makeups were predisposed to form teratomas with or without somatic transformation, and we correlated those findings with clinical outcome. The data from RPLND that had been performed before chemotherapy for the purposes of diagnosis, staging, or therapy were analyzed separately. Salvage chemotherapy was defined as the receipt of any second-line chemotherapy for progressive or relapsed disease, usually given after first-line treatment (ie, bleomycin, etoposide, and cisplatin or etoposide and cisplatin). A change in chemotherapy regimen to consolidate a complete response was not considered salvage therapy. Adjuvant chemotherapy was also not counted as salvage therapy in the analysis.

Patients' pathologic reports, laboratory test results, and clinical histories were collected from MD Anderson's clinical data-management computer system. The dates of patients' deaths were obtained from their medical records or from the Social Security Death Index (available at: http://ssdi.genealogy.rootsweb.com/, accessed March, 2015). The principal endpoint for this study was cancer-specific mortality. Patients with no evidence of active NSGCT who died as a result of other causes, such as treatment-related complications, accidents, or comorbidities, were included in the analysis of cancer-specific mortality. Survival duration was measured from the date of diagnosis to the date of death or the most recent date of record if the patient was still alive. For the 6 patients with metachronous tumors, the survival duration was measured from the date of first diagnosis of NSGCT. If pathologic reports from both MD Anderson and an outside institution were available, then the report from MD Anderson was used to maximize consistency.

Exome Sequencing

Between June 2014 and February 2015, 11 patients with progressive or relapsed NSGCT were prospectively enrolled in a laboratory protocol to sequence the entire coding region of 409 genes in tumor and paired germline tissues (the Cancer Mutation Scan 400 [CMS-400] panel). The eligibility criteria for the study included refractory disease that required novel therapy in a clinical trial, an Eastern Cooperative Oncology Group performance status <1, and a creatinine level ≤ 2.0 mg/dL.

Pathologists in the Tissue Qualification Laboratory identified the optimal formalin-fixed, paraffin-embedded tissue blocks for the study. For each paraffin block, a hematoxylin and eosin (H&E)-stained slide and unstained sections were prepared. The tumor tissue was dissected from an unstained sequential section using the H&E slide as a template. DNA was then extracted from the dissected tumor using the QIAamp DNA FFEP Tissue Kit (Qiagen Inc, Valencia, Calif) and was used to sequence genes in a CMS-400 panel (Ion Proton System; Life Technology/Thermo Fisher Scientific, Inc, Waltham, Mass). All procedures were well established for the testing of solid tumors.[10]

Statistical Considerations

We estimated the cumulative incidence functions for NSGCT-related death, treating non-NSGCT–related death as a competing risk.[11] The differences between these functions were assessed using the Gray test for cause-specific death. Because of its bias for cancer-specific death due to competing risks from death without cancer, we did not use the Kaplan-Meier method in our calculations. A Fine and Gray proportional hazards regression analysis was used to assess the relations between study factors and NSGCT-related death while treating non-NSGCT–related death as a competing risk.[12] The Pearson chi-square test and the Fisher exact test were used to compare proportions between independent samples.

Assuming that the incidence rate of a particular causative genetic mutation is high in a relatively homogeneous subgroup and low in the entire cancer population,[13] we expected that the incidence rate of a driver mutation would be at least 60% in a potentially lethal phenotype within the mixed yolk sac and yolk sac-seminoma subgroups. The exact binomial test was used to determine whether a genetic mutation was causal or incidental. Exact binomial (Clopper-Pearson) 95% confidence intervals were also computed.

All statistical analyses were performed using TIBCO Spotfire S + 8.2 software for Windows (TIBCO Spotfire, Boston, Mass) and StatXact-9 (Cytel Software Corporation, Cambridge, Mass). This study (PA14-0099 and PA14-0894) was approved by the MD Anderson institutional review board.

RESULTS

We identified 703 patients with NSGCT who had been diagnosed from January 2000 to December 2010. We excluded 88 patients on the basis of the following criteria: orchiectomy after chemotherapy (50 patients), pathologic data not available (22 patients), pure seminoma (8 patients), nongerm cell tumor (eg, paratesticular tumor; 4 patients), age <3 years with pure yolk sac tumor or teratoma (3 patients), and extragonadal germ cell tumor (1 patient). We included 7 patients whose primary tumors were identified as pure seminoma or "burnt-out" tumor but who had elevated serum α-fetoprotein levels, indicating that the tumors were NSGCT.

Table 1 lists the clinical characteristics of the 615 patients who were included in the study and the pathologic properties of their 621 primary testicular tumors (6 patients had metachronous NSGCT). Nine percent of patients had died by 10 years: 7% from NSGCT and 2% from noncancer-related causes, such as chemotherapy toxicities, surgical complications, accidents, and comorbid conditions.

The results of our multivariate analyses indicated that the presence of yolk sac tumor in the primary tumor ($P = .0003$) was associated with a high cancer-specific mortality rate. The clinical stage at the time of diagnosis was also a significant prognostic factor (stage IIIC; $P < .0001$). However, the size of the primary tumor and the presenting level of human chorionic gonadotropin or α-fetoprotein were not significant. Supporting Figure 1 (see online supporting information) shows that both the presence (Supporting Fig. 1A) and the proportion (Supporting Fig. 1B) of yolk sac tumor in the primary tumor were associated with a higher cancer-specific mortality rate.

There were 5 distinct subgroups of NSGCT: embryonal (n = 111), mixed choriocarcinoma (n = 65), yolk sac-seminoma (n = 95), mixed yolk sac (n = 241), and mixed seminoma (n = 99; $P = .002$), as illustrated in Figures 1 and 2. The results of a multivariate analysis indicated that tumors in the yolk sac-seminoma ($P = .007$) and mixed yolk sac ($P < .05$) subgroups were associated with an unfavorable cancer-specific mortality rate (Table 2).

Table 3 provides data on the histologic makeup of the 5 NSGCT subgroups. The 5-year cancer death cumulative incidence (CDCI) and the 10-year CDCI of patients within each subgroup are provided in Supporting

TABLE 1. Clinical and Tumor Characteristics of Patients With Nonseminomatous Germ Cell Tumor of the Testis

Characteristic	No. of Patients (%)
Total patients	615 (100)
Age: Median [range], y	27 [12-70]
Race	
White	416 (68)
Hispanic	176 (29)
African American	14 (2)
Asian	9 (1)
Pathology of primary tumor	
Mixed germ cell tumor	509 (83)
Embryonal carcinoma	68 (11)
Teratoma	21 (3)
Yolk sac tumor	6 (1)
Choriocarcinoma	4 (1)
Atypical seminoma	3 (1)
Burned-out primary	4 (1)
Stage[a]	
IA	162 (26)
IB	100 (16)
IS	30 (3)
IIA	63 (10)
IIB	52 (8)
IIC	22 (4)
IIIA	57 (9)
IIIB	64 (10)
IIIC	71 (11)
Size of primary tumor: Median [range], cm	3.5 [0-27]
Therapy	
Salvage chemotherapy	73 (12)
High-dose chemotherapy with transplantation support	15 (2)
Whole-brain radiation	9 (1)
RPLND	212 (34)
Teratoma	107 (17)
Somatic transformation	20 (3)
Viable germ cell tumor	27 (4)
No evidence of disease	58 (9)
Died	57 (9)
NSGCT	45 (7)
Unrelated	12 (2)
MDS/AML	2 (<1)
Chemotherapy toxicities	3 (1)
Surgical complications	1 (<1)
Accidents	1 (<1)
Comorbidities	2 (<1)
Unknown	3 (1)

Abbreviations: MDS/AML, myelodysplastic syndrome/acute myelogenous leukemia; NSGCT, nonseminomatous germ cell tumor; RPLND, retroperitoneal lymph node dissection; SMT, Shi-Ming Tu.
[a] Six patients had bilateral metachronous NSGCT.

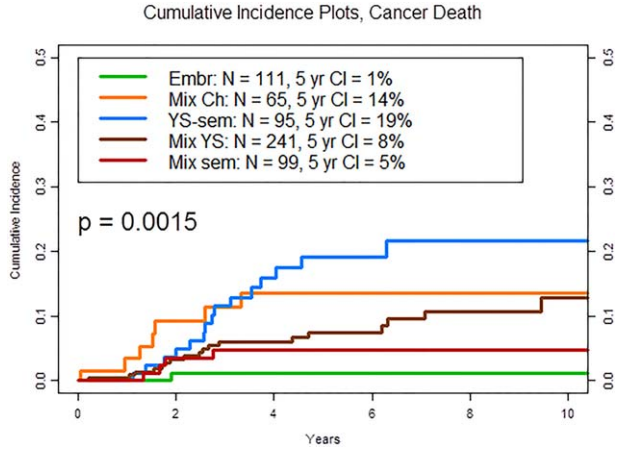

Figure 1. This is a plot of the cumulative incidence of cancer death in the subgroups with embryonal (Embr) (green), mixed choriocarcinoma (Mix Ch) (orange), mixed yolk sac (Mix YS) (brown), yolk sac-seminoma (YS-sem) (blue), and mixed seminoma (Mix sem) (purple) tumors according to the histologic makeup of the primary tumor. CI indicates confidence interval.

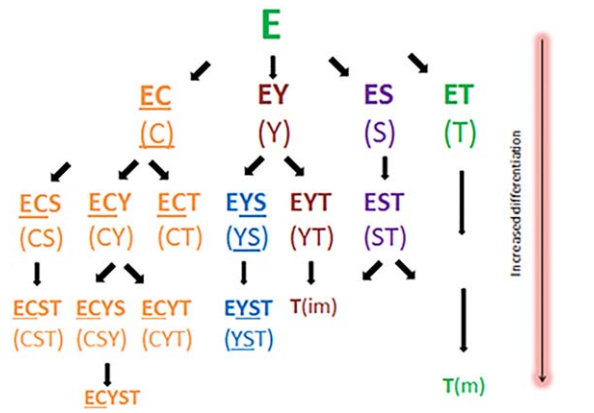

Figure 2. Subgroups with nonseminomatous germ cell tumor of the testis are illustrated on the basis of histologic makeup, clinical characteristics, and putative developmental origin (see Masters JR, Koberle B. Curing metastatic cancer: lessons from testicular germ-cell tumors. *Nat Rev Cancer.* 2003;3:517-525[2]). Embryonal (green), mixed choriocarcinoma (orange), mixed yolk sac (brown), yolk sac-seminoma (blue), and mixed seminoma (purple). E indicates embryonal; C, choriocarcinoma; Y, yolk sac tumor; S, seminoma; T, teratoma; T(im), immature teratoma; T(m), mature teratoma.

Table 1 (see online supporting information). There was no consistent trend in clinical outcomes over time. The 5-year CDCI by year was 12% in 2000, 8% in 2001, 8% in 2002, 4% in 2003, 10% in 2004, 10% in 2005, 12% in 2006, 4% in 2007, 9% in 2008, and 17% in 2009.

An intriguing finding was that somatic transformations occurred more commonly in metastatic lesions in the yolk sac-seminoma subgroup (Supporting Table 2; see online supporting information): they were identified in 13 of those 95 patients (14%) versus only 8 of the 516 patients (1.6%) in other subgroups ($P < .0001$). However, somatic transformations occurred at the same rate in the primary tumor in all subgroups. Fifteen of the 21 patients (71%) who had somatic transformation in metastatic lesions died of their NSGCT compared with 0 of 15 patients (0%) who had transformations in the primary tumor.

TABLE 2. Fine and Gray Proportional Hazards Regression Analysis of Death From Cancer in Patients With Nonseminomatous Germ Cell Tumor of the Testis (n = 615)

Variable[a]	HR (95% CI)	*P*
Subgroup		
Embryonal	1.0	
Mixed choriocarcinoma	7.2 (0.8-62)	.071
Yolk sac-seminoma	17 (2.2-128)	.0066
Mixed yolk sac	7.6 (1.0-57)	.049
Mixed seminoma	3.8 (0.4-34)	.23
Age, y		
12-40	1.0	
41-71	1.6 (0.8-3.2)	.18
Stage		
IS-IIA	1.0	
IIB-IIIB	4.9 (1.9-12)	.0008
IIIC	18 (7.2-44)	< .0001

Abbreviations: CI, confidence interval; HR, hazard ratio.
[a] Other variables, such as the size of the primary tumor and the presenting human chorionic gonadotropin or α-fetoprotein levels, were not significant in separate analyses;

TABLE 3. Intratumoral Heterogeneity in Patients With Nonseminomatous Germ Cell Tumor of the Testis: Histologic Makeup and Clinical Stage

		Clinical Stage: No. of Patients			
Subgroup: Histologic Makeup	No. of Patients (%)	IIIC	IIIB	IIIA	I-II
Embryonal	112 (18)	6	10	17	79
E	68 (11)	3	7	9	49
E+T	44 (7)	3	3	8	30
Mixed choriocarcinoma	65 (10)	21	12	4	28
C	3 (<1)	3	0	0	0
E+C	3 (<1)	1	1	0	1
E+C+Y	5 (1)	1	0	1	3
C+Y	1 (<1)	1	0	0	0
E+C+S	2 (<1)	0	1	0	1
C+S	2 (<1)	0	1	1	0
E+C+T	8 (1)	3	0	0	5
C+T	2 (<1)	2	0	0	0
E+C+Y+T	25 (4)	4	8	2	11
C+Y+T	2 (<1)	1	0	0	1
E+C+Y+S	1 (<1)	1	0	0	0
C+S+T	4 (1)	2	1	0	1
E+C+Y+S+T	7 (1)	2	0	0	5
Yolk sac-seminoma	96 (15)	9	6	9	72
E+Y+S	24 (4)	3	1	5	15
Y+S	3 (<1)	1	0	0	2
E+Y+S+T	58 (9)	5	4	4	45
Y+S+T	11 (2)	0	1	0	10
Mixed yolk sac	243 (39)	26	21	18	178
Y	6 (1)	2	2	1	1
E+Y	49 (8)	5	5	4	35
E+Y+T	149 (24)	12	9	9	119
Y+T	18 (3)	3	2	1	12
T	21 (3)	4	3	3	11
Mixed seminoma	101 (16)	8	13	9	71
S	3 (<1)	1	2	0	0
E+S	52 (8)	2	6	6	38
E+S+T	19 (3)	1	3	2	13
S+T	27 (4)	4	2	1	20
None[a]	4 (1)	1	2	0	1
Total	621[b]	71	64	57	429

Abbreviations: C, choriocarcinoma; S, seminoma; E, embryonal carcinoma; Y, yolk sac tumor; T, teratoma.
[a] Four patients had burnt-out primary tumors.
[b] Six patients had bilateral metachronous NSGCT.

We also investigated whether patients who had low-stage but high-risk disease were likely to die of their NSGCT. Patients with clinical stage I or II disease in the yolk sac-seminoma and mixed yolk sac subgroups had statistically significantly higher CDCIs than did those in the other subgroups ($P < .05$) (Supporting Fig. 2A; see online supporting information). Of the 14 patients with clinical stage I or II disease who died of their NSGCTs, 13 (93%) were in the yolk sac-seminoma or mixed yolk sac subgroup.

Eleven patients with refractory NSGCT were enrolled in a prospective laboratory study in which next-generation sequencing was performed on their residual metastatic tumors after chemotherapy. All patients had yolk sac tumor in their primary or metastatic tumors. Four patients had experienced progression on high-dose chemotherapy with transplantation support. Six patients had died. Another 4 patients were undergoing experimental treatment or were in hospice care.

We sequenced the entire coding region of 409 genes in refractory, metastatic NSGCTs from 9 patients with a yolk sac tumor phenotype (Table 4). Two patients with extragonadal germ cell tumors were not included in this analysis. Six patients had yolk sac-seminoma (2 with embryonal [E], yolk sac tumor [Y], seminoma [S], and teratoma [T] [EYST] components and 1 with EYS components) or mixed yolk sac (1 each with EYT, YT, and T components) NSGCT. The remaining 3 patients had primary (1 with ECYT components) or metastatic yolk sac tumors. None of the 21 somatic mutations detected were shared among the 9 patients. Two different mutations involving the same gene (ie, catenin β 1 [*CTNNB1*] and serine/threonine kinase 36 [*STK36*]) were observed in 2 of 9 patients, respectively. If the expected incidence rate of a driver mutation in a potentially lethal NSGCT subtype with a yolk sac tumor component is 60%, then the observed incidence rates of 0% (0 of 9 patients; 95% confidence interval, 0%-34%) for a specific mutation ($P = .0003$) and 22% (2 of 9 patients; 95% confidence interval, 3%-60%) for a mutated gene (eg, *CTNNB1* or *STK36*; $P = .035$) suggest that the mutation or the mutated gene is incidental rather than causal.

TABLE 4. Molecular Profile of Refractory Nonseminomatous Germ Cell Tumor of the Testis With a Yolk Sac Tumor Phenotype

Patient	Gene	Standardized Nomenclature (HGVS)	Location	DNA Change	Protein Change	Histologic Makeup
1[a]	PTEN	NM_000314.4(PTEN):c.609_625del p.I203fs*34	Exon 6	Deletion	Frameshift	E+S+Y+T
	EP300	NM_001429.3(EP300):c.1876C>T p.R626*	Exon 9	SNV	Nonsense	
	STK36	NM_015690.4(STK36):c.127_129del p.K43del	Exon 3	Deletion	Deletion	
2[b,c]	None					E+S+Y+T
3[a]	ARID1A	NM_006015.4(ARID1A):c.2474G>A p.S825N	Exon 8	SNV	Missense	E+S+Y
	DST	NM_001723.5(DST):c.6880A>C p.N2294H	Exon 24	SNV	Missense	
4[b]	ERBB3	NM_001982.3(ERBB3):c.850G>A p.G284R	Exon 7	SNV	Missense	E+Y+T
	LRP1B	NM_018557.2(LRP1B):c.2131T>A p.W711R	Exon 13	SNV	Missense	
	PBRM1	NM_018313.4(PBRM1):c.1541G>T p.S514I	Exon 14	SNV	Missense	
5[b]	CTNNB1	NM_001904.3(CTNNB1):c.999C>G p.Y333*	Exon 7	SNV	Nonsense	Y+T
		NM_001904.3(CTNNB1):c.1055G>A p.S352N	Exon 7	SNV	Missense	
6[a]	CTNNB1	NM_001904.3(CTNNB1):c.134C>T p.S45F	Exon 3	SNV	Missense	T
	BRIP1	NM_032043.2(BRIP1):c.3488A>C p.D1163A	Exon 20	SNV	Missense	
	EP400	NM_015409.4(EP400):c.5170C>T p.R1724C	Exon 27	SNV	Missense	
	PDE4DIP	NM_014644.4(PDE4DIP):c.1523A>T p.Q508L	Exon 12	SNV	Missense	
7[b,c]	EGFR	NM_005228.3(EGFR):c.2716G>T p.E906*	Exon 23	SNV	Nonsense	E+C+Y+T
	IGF1R	NM_000875.3(IGF1R):c.2186C>T p.S729F	Exon 10	SNV	Missense	
	UBR5	NM_015902.5(UBR5):c.6056A>G p.N2019S	Exon 43	SNV	Missense	
8	MLL3	NM_170606.2(MLL3):c.6027A>T p.K2009N	Exon 36	SNV	Missense	Y
	AFF1	NM_005935.2(AFF1):c.3490G>A p.A1164T	Exon 19	SNV	Missense	
	ICK	NM_016513.4(ICK):c.1423C>T p.R475W	Exon 12	SNV	Missense	
9	MTRR	NM_024010.2(MTRR):c.1084_1085delinsTT p.D362F	Exon 7	Indel	Missense	Y
	STK36	NM_015690.4(STK36):c.1291C>T p.P431S	Exon 11	SNV	Missense	

Abbreviations: AFF1, AF4/FMR2 family member 1; ARID1A, AT-rich domain 1A; BRIP1, BRCA1 interacting protein C-terminal helicase 1; C, choriocarcinoma; c., codon; CTNNB1, catenin β 1; DST, dystonin; E, embryonal carcinoma; EGFR, epidermal growth factor receptor; EP300, E1A binding protein p300; EP400, E1A binding protein p400; ERBB3, erb-b2 receptor tyrosine kinase 3; HGVS, Human Genome Variation Society; ICK, intestinal cell kinase; IGF1R, insulin-like growth factor 1 receptor; Indel, insertion-deletion; LRP1B, low-density lipoprotein receptor protein 1B; MLL3, myeloid/lymphoid leukemia 3 (lysine methyltransferase 2C); MTRR, 5-methyletrahydrofolate-homocysteine methyltransferase reductase; PBRM1, protein polybromo-1; PDE4DIP, phosphodiesterase 4D interacting protein; PTEN, phosphatase and tensin homolog; S, seminoma; SNV, single nucleotide variant; STK36, serine/threonine kinase 36; T, teratoma; UBR5, ubiquitin protein ligase E3 component n-recognin 5; Y, yolk sac tumor.

[a] This patient had somatic transformation.

[b] This patient died.

[c] This patient had disease progression while receiving high-dose chemotherapy and transplantation support.

DISCUSSION

In this study, we investigated the role of differentiation in a potentially lethal subtype of NSGCT. We demonstrated that, despite intratumoral heterogeneity, complex or mixed tumors are still very curable with integrated or multimodal therapy. Hence, the embryonal subtype is very amenable to chemotherapy, whereas certain yolk sac tumors need both chemotherapy and surgery to be cured. When clinical outcome is related to a particular disease rather than a specific treatment, identifying the disease subtypes may enable improved patient selection and personalized care.

We identified 5 subgroups of NSGCT on the basis of their histologic makeup, clinical characteristics, and putative cellular origins (Fig. 2). This schema recapitulates the developmental and differentiation pathways of embryonic germ cells.[2]von Hochstetter and Hedinger[14] and Jacobsen et al[15] evaluated the distribution of diverse histologic components in NSGCT. However, those authors did not elaborate on the presence of yolk sac tumor in mixed NSGCT. The results of several reports have indicated that NSGCTs with yolk sac tumor components have a poorer prognosis than those without yolk sac components.[16-19] Furthermore, a higher frequency of yolk sac tumor was detected at autopsy during the chemotherapy era than during the prechemotherapy era.[20]

In many respects, the histopathology of yolk sac tumors encapsulates the histology of the yolk sac. Hence, yolk sac tumors represent a spectrum of tumors that encompass all possible phenotypes derived from endodermal and mesenchymal differentiation. Talerman recognized 9 different patterns of yolk sac tumor,[17] whereas Ulbright et al described 11 (reticular, macrocytic, endodermal sinus, papillary, solid, glandular-alveolar [including intestinal and endometrioid-like], myxomatous, sarcomatoid, polyvesicular vitelline, hepatoid, and parietal).[21] Nogales et al suggested that yolk sac tumors arise from pluripotent malignant stem cells.[22] In addition, Marin-Padilla predicted the presence of, and Susuki et al detected, tumor-initiating cancer stem cells in ovarian yolk sac tumors.[23,24]

The results from this study suggest that the histologic makeup of primary NSGCTs provides useful prognostic information and has profound therapeutic implications. Both the presence and a high proportion of yolk sac tumor component were associated with an unfavorable prognosis (Supporting Fig. 1; see online supporting information). In an era of effective chemotherapy, it is not surprising that the presence of indolent phenotypes such as yolk sac tumor imparts high drug resistance and a low cure rate.[25] Moreover, certain phenotypes, such as choriocarcinoma and seminoma, behave differently when they present as pure or mixed tumors. Therefore, not only does the composition of NSGCT allude to its innate biologic behavior, but the interactions among the various histologic constituents affect its ultimate clinical course.

It remains unclear whether certain histologic makeups predispose NSGCTs to somatic transformation and increase their lethality. Currently, malignant somatic transformation is believed to arise from a preexisting teratomatous component of NSGCT. Alternatively, it may evolve from pluripotent malignant stem cells. Because some patients who developed malignant somatic transformation never had teratomas, it was thought that yolk sac tumor was a plausible origin. To our knowledge, our data are the first to demonstrate that yolk sac-seminoma tumors are predisposed to undergo somatic transformation and may increase the risk of death from NSGCT ($P < .0001$) (Supporting Table 2; see online supporting information). This finding is novel and may be paradigm-shifting if not practice-shifting. However, the results need to be validated in another independent database.

We note that our data suggest that certain patients with clinical stage I and II yolk sac-seminoma or mixed yolk sac tumors are at risk of dying from their NSGCT. Of the 14 patients with clinical stage I and II disease who died, 13 (93%) were in the yolk sac-seminoma or mixed yolk sac subgroup (Table 3). Six of those patients (43%) had experienced somatic transformation of their tumors during their clinical course. It is also noteworthy that many patients (10 of 14; 71%) either did not undergo surgical intervention to remove residual tumor tissue after completing chemotherapy or had psychosocial issues (eg, denial, substance abuse, no insurance coverage, or financial burden) that led to noncompliance with treatment and might have contributed to their death. It is imperative that we identify such patients for whom the window of opportunity for cure is narrow, because their NSGCT may be less amenable to surgery and more resistant to chemotherapy as it becomes advanced and systemic.

This study provides a critical observation and a cautionary notice about the relevance of intratumoral heterogeneity and precision medicine in patients with NSGCT in particular and those with solid tumors in general.[26] Because embryonal carcinoma is exquisitely chemosensitive and teratoma is completely chemoresistant, it is necessary to integrate chemotherapy with surgery to cure mixed NSGCTs. However, when the different histologic components of NSGCTs have a common clonal origin and are known to harbor a similar, if not identical, genetic profile (eg, both chemosensitive embryonal carcinoma and chemoresistant teratoma have wild-type tumor protein 53 [*TP53*] or defective B-cell lymphoma 2 [*BCL-2*]), it is unlikely that targeting the same genetic defects in such disparate cellular phenotypes will significantly affect the overall clinical course of the disease.[27,28] Although the genes are important, the type of cells in which they exert their influence is paramount.[3,29]

NSGCT is known to have a markedly low mutation rate because of its embryonic origins.[30] In a relatively simple disease entity, we postulated that it would be easy to discover meaningful genetic targets for diagnosis, prognosis, and therapy and to identify a potentially lethal subgroup of NSGCTs. Although we did not detect any consistent mutations in a limited panel of putative, actionable, and supposedly pertinent genes, additional or unknown genetic aberrations and epigenetic abnormalities could still be "drivers" in the pathogenesis of a particular subtype of NSGCT.[31]

Our data support the notion that intratumoral heterogeneity is caused in part by differentiation of pluripotent progenitor cells.[3,4,32] Notably, intratumoral heterogeneity at the cellular level revealed distinct tumor subtypes and developmental processes that can be targeted in precision medicine. Furthermore, a cellular origin could account for the many aspects of intratumoral heterogeneity, including truncal and branched driver events, clonal and subclonal mutations, parallel evolution, epistatic interactions, pathogenic and nonpathogenic mutations, temporal and spatial diversity, and genetic instability.[26] Our results suggest that a cellular profile is more useful than a genetic target for the design of precision medicine. Although a genetic origin of cancer is undisputed, a cellular origin is key to framing the correct hypotheses, designing informative experiments, and discovering effective treatments.[33] If confirmed, the results of this study will have major clinical implications concerning the future direction and eventual fulfillment of precision medicine in cancer care.

In summary, NSGCT comprises distinct subtypes with unique clinical features and biologic characteristics. The presence of yolk sac tumor in a primary tumor may

adversely affect the prognosis of patients with NSGCT by predisposing the tumor to somatic transformation. Our data suggest that intratumoral heterogeneity can be traced to the differentiation of pluripotent progenitor cells and may be useful for identifying a potentially lethal subtype of NSGCT. The results of this study need to be validated in another database study or a prospective clinical trial.

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