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Original contribution

High-resolution telomere fluorescence in situ hybridization reveals intriguing anomalies in germ cell tumors☆,☆☆

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Summary Testicular germ cell tumor (TGCT) is the most common malignancy of young men. Most patients are completely cured, which distinguishes these from most other malignancies. Orchiectomy specimens (n = 76) were evaluated using high-resolution (single-cell discriminative) telomere-specific fluorescence in situ hybridization (FISH) with simultaneous Oct4 immunofluorescence to describe telomere length phenotype in TGCT neoplastic cells. For the first time, the TGCT precursor lesion, germ cell neoplasia in situ (GCNIS) is also evaluated in depth. The intensity of the signals from cancerous cells was compared to the same patient's reference cells—namely, healthy germ cells (defined as "medium" length) and interstitial/somatic cells (defined as "short" telomere length). We observed short telomeres in most GCNIS and pure seminomas (P = .006 and P = .0005, respectively). In contrast, nonseminomas displayed longer telomeres. Lesion-specific telomere lengths were documented in mixed tumor cases. Embryonal carcinoma (EC) demonstrated the longest telomeres. A fraction of EC displays the telomerase-independent alternative lengthening of telomeres (ALT) phenotype (24% of cases). Loss of ATRX or DAXX nuclear expression was strongly associated with ALT; however, nuclear expression of both proteins was retained in half of ALT-positive ECs. The particular distribution of telomere lengths among TGCT and GCNIS precursors implicate telomeres anomalies in pathogenesis. These results may advise management decisions as well.
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1. Introduction

Testicular germ cell tumor (TGCT) is the most common malignancy of young men between the ages of 15 and 35 years [1]. TGCT subtypes are classified as seminomatous or nonseminomatous. The only "seminomatous" lesion is seminoma (SEM). nonseminomas (NSs) include embryonal carcinoma (EC), teratoma (TER), yolk sac tumor (YST), and choriocarcinoma. Some patients present with a mixed germ cell tumor (MGCT) phenotype, where 2 or more subtypes of TGCT are present. TGCTs are one of the few exquisitely treatable cancer types. Relatively little is known about TGCT pathogenesis. In particular, telomere biology in TGCT initiation and progression has been minimally explored. Germ cell neoplasia in situ (GCNIS), the precursor lesion of TGCT, is newly characterized here in the context of telomeres. A better understanding of molecular pathogenesis should explain why TGCTs are more responsive to treatment and whether this could be applied to more lethal somatic cancers.

Telomeres are nucleic acid repeats at chromosome ends progressively lost at every cell division cycle. This "attrition" limits cellular replicative potential while preserving DNA integrity. Telomeric DNA consists of tandem repeats of TTAGGG noncoding sequence. In healthy somatic cells, telomere attrition acts as a tumor suppressor by activating senescence or apoptosis programs. If these "failsafes" become abrogated, excessive telomere shortening generates loss of genomic integrity. Cells survive by activating the telomere-synthesizing enzyme telomerase (TERT) and become immortalized [2,3]. Many epithelial somatic tumors display short telomeres and increased telomerase [4–8]. Five percent of cancers maintain their telomeres via the telomerase-independent alternative lengthening of telomeres (ALT) pathway [9]. ALT uses homologous recombination as telomeres of adjacent chromosomes are haphazardly "copy-pasted" onto one another. This creates telomere length heterogeneity and is associated with nuclear loss of ATRX or DAXX proteins or mutations in histone variant H3.3 (ie, either *H3F3B* or *H3F3A* genes) [10]. ATRX and DAXX are H3.3 chaperones that interact to form a chromatin remodeling complex at telomeres [11].

Using a telomere-specific fluorescence in situ hybridization (FISH) method applied to fixed tissue specimens, we previously conducted a survey of the ALT across cancers, including few TGCT cases [9]. ALT was absent in SEM, but it was detected in 15% of NS.

Here, we report an elaborate study focused on comparing TGCT neoplastic cell telomere lengths, including GCNIS precursor, with reference cell comparators within the same patient. Also, we provide a precise TGCT subtype-specific determination of the prevalence of the ALT phenotype.

Table 1 Study cohort patient characteristics and clinical data

	EC	SEM	TER	YST	Mixed
n	7	43	3	3	20
Mean age (y)	24.7	36.4	20.3	33	27.4
Ethnicity					
White (%)	100	86.1	100	33.3	70
Nonwhite (%)	0	11.6	0	66.7	20
Unknown ethnicity (%)	0	2.3	0	0	10
Stage					
I	71.4	95.3	100	100	70
II	28.6	4.7	0	0	30

2. Materials and methods

2.1. Case selection and tissue microarray layout

Formalin-fixed, paraffin-embedded tissues from 76 individual TGCT cases treated by orchiectomy at the Johns Hopkins Hospital from 1995 to 2008 were analyzed. All data and specimen collection was performed in compliance with institutional review board protocol and international standards of protection of research subjects and patient privacy laws. Twenty of the 76 cases were MGCTs. The following were excluded from this study: pediatric YST, spermatocytic SEMs, primary tumors at ectopic sites (eg, mediastinum or brain), lymph node metastatic lesions, and Sertoli or Leydig cell tumors. Moreover, choriocarcinomas were not available for study. Clinical characteristics of the study cohort are shown in Table 1. Tissue microarrays (TMAs) were constructed from the original formalin-fixed, paraffin-embedded blocks. For each case, areas of tumor, GCNIS (when present), and healthy tissue were identified from hematoxylin and eosin–stained sections by the pathologist. The marked areas were then used to harvest paraffin cores to assemble the TMA. All lesions were represented by 3 replicate cores, each measuring approximately 0.6 mm in diameter. TMAs were labeled as described below and then histologically scored. Of 76 total patient specimens, 70 were arrayed on the TMA. The remaining 6 were analyzed by study of whole sections.

2.2. FISH and immunofluorescence staining

Briefly, slides were deparaffinized by first heating to 62°C on a hotplate for 10 minutes, immersion in 3 consecutive tanks of xylene for 5 minutes each, hydrating through a graded ethanol series, placed in citrate buffer, and steamed for 25 minutes. A Cy3-labeled, telomere-specific, 18-mer peptide nucleic acid hybridization probe (Panagene, Inc, Daejeon, South Korea; N-to-C terminus sequence: CCCTAACCCTAACCCTAA) of 300 ng/mL specific for the telomere DNA repeat sequence, dissolved in hybridization buffer (70% formamide, 10 mM Tris [pH 7.5], in ultrapure DNAse-free water), was applied to the slides and co-denaturation was performed by incubation for 5 minutes at 84°C. In addition, Alexa Fluor 488–labeled, centromere-specific FISH probe was included as a control of hybridization efficiency (N-to-C terminus sequence: ATTCGTTGGAAACGGGA). Slides were hybridized with these probes for

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