The Role of the Mouse Y Chromosome on Susceptibility to Testicular Germ Cell Tumors

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Abstract

Testicular germ cell tumors (TGCT) are sex limited, occurring only in males with a Y chromosome. Recently, the gr/gr deletion on the human Y chromosome was associated with increased risk of TGCTs. In addition, the presence of Y chromosome sequences is associated with TGCTs in cases of gonadal dysgenesis. TGCTs in strain 129 males recapitulate many aspects of testicular cancer in human infants and can be used to evaluate the role of the Y chromosome in TGCT risk. We used chromosome substitution strains and a sex-reversing mutant to test the role of the Y chromosome on TGCT susceptibility. Our results show that a Y-linked gene that does not differ among the tested strains is essential for tumorigenesis. [Cancer Res 2009;69(8):3614–8]

Introduction

Testicular cancer is the most common malignancy affecting young men ages 15 to 34 and accounts for more than 1% of all male cancers (1, 2). Unfortunately, testicular germ cell tumor (TGCT) rates have been rising worldwide in the past several decades, making it important to understand the etiology and pathogenesis of TGCTs. Family history is a significant risk factor, with the likelihood of developing TGCTs 8- to 10-fold higher among brothers and 4-fold higher among sons of affected individuals (3, 4). Although high heritability suggests a strong genetic component to susceptibility, only one low penetrance susceptibility variant has been found.

Growing evidence suggests that genetic factors on the Y chromosome are involved in the development of TGCTs. Recently, a 1.6-Mb deletion within the AZFc (azoospermia factor) region on Yq11 was associated with increased risk of TGCTs in hemizygous men (5, 6). This deletion, called gr/gr, confers a 1.5-fold increased risk in men without a family history of TGCT and a 2.3-fold increased risk in men with a family history of TGCT, and is present in 1% of unaffected individuals (5). The gr/gr deletion results in loss of three genes (DAZ, BPY2, and CDY1), all of which are expressed in spermatogonia (7–9), suggesting that at least one of these genes normally suppresses TGCT susceptibility. The AZF region has also been implicated in male factor infertility (10), which is another risk factor for TGCTs (11, 12). Structural abnormalities of the Y chromosome are a major cause of male factor infertility (13, 14). The strong association between reduced fertility and TGCTs suggests that they share genetic and developmental determinants (15).

Studies of patients with gonadal dysgenesis provide further evidence for involvement of the Y chromosome with TGCTs. Among affected individuals, those with a normal 46 XY karyotype show increased risk of TGCT relative to those with a 45 XO karyotype (16–19), suggesting that at least one genetic factor on the Y chromosome promotes TGCTs. Males with loss of some or all of the Y chromosome could be used to clarify the role of the Y chromosome in TGCT risk. However, individuals with DNA sequence variants or chromosome rearrangements including deletions are uncommon, making a mouse model relevant for testing the role of the Y chromosome on TGCT susceptibility.

Strain 129 mice spontaneously develop TGCTs at a rate of 1% to 8%, depending on the substrain (20, 21). These TGCTs are an established model of testicular teratomas and teratocarcinomas of infants. TGCTs in infants and mice both arise from primordial germ cells (PGCs) and lack both intratubular germ cell neoplasia (carcinoma in situ) and characteristic karyotypic abnormalities (e.g., iso12p) found in adult TGCTs in humans.

As in humans, control of TGCT tumorigenesis in mice is complex. Crosses between 129/Sv and seven other inbred strains yielded a single affected mouse among 11,292 male mice surveyed, suggesting multigenic control (22). In mice, only one susceptibility gene, the Pten (phosphatase and tensin homologue) tumor suppressor, has been reported (23, 24).

Given this background, we evaluated the role of the Y chromosome on TGCT susceptibility by testing whether the Y chromosome from the 129/Sv strain is sufficient for tumorigenesis by measuring the frequency of affected males in the B6-Chr Y129 chromosome substitution strain (CSS). CSSs are inbred strains in which a chromosome in the host strain has been replaced by the corresponding chromosome from a donor strain (25, 26). For example, the B6-Chr Y129 CSS substitutes the 129/Sv donor Y chromosome on an otherwise inbred C57BL/6j background. We also tested whether the 129/Sv-derived Y chromosome is necessary for TGCT tumorigenesis by measuring the frequency of affected males in 129-Chr Y129 and 129-Chr Y129/129 CSSs.

In addition, we used a sex-reversing mutant, Odd Sex (Ods), to test whether any Y chromosome is required for tumorigenesis. Ods is a dominant insertional mutant that causes complete female-to-male sex reversal in the absence of the master sex-determining gene, Sry (27). Apart from the testes, sex-reversed mice are phenotypically similar to their normal XY littermates. In the testes of sex-reversed mice, germ cells are present during the critical period of TGCT formation but are lost soon after birth. Sex-reversed mice therefore provide an opportunity to test for initiation of TGCTs in the absence of the Y chromosome. Results of both studies of CSS and sex-reversed males suggest that a Y-linked gene
that does not differ among the tested strains is essential for tumorigenesis.

Materials and Methods

**CSS mice.** 129S1/Svvlm (129/Sv), C57BL/6j (B6), and MOLF/Eij (MOLF) were purchased from The Jackson Laboratory. C57BL/6j-Chr Y^{129S1/Svvlm}/Na (B6-Chr Y^{129}) was made by backcrossing (C57BL/6j × 129S1/Svvlm) F1 males to C57BL/6j females to generate N2 males, which were then backcrossed to C57BL/6j females to generate N3 males. These backcrosses were continued until the N10 generation and the CSS was maintained thereafter by brother-sister mating. No genotyping of backcross males was necessary because the nonrecombinant portion of the Y chromosome is transmitted intact from fathers to sons. Similar backcrosses were used to make the 129S1/Svvlm-Chr Y^{MOLF/Eij}/Na (129-Chr Y^{MOLF/Eij}) CSS. The 129S1/Svvlm-Chr Y^{C57BL/6j}/Na (129-Chr Y^{B6}) has been described (28). All data reported from CSS males were from the N10 backcross generation or later. Mice were housed in the Case Western Reserve University (CWRU) Animal Resource Center and maintained on a 12:12-h light/dark cycle. Mice were given water and LabDiet 5010 chow (PMI Nutrition International) and testes were examined for TGCTs. Segregation and TGCT data were reported from CSS males were from the N10 backcross generation or later. Mice were housed in the Case Western Reserve University (CWRU) Animal Resource Center and maintained on a 12:12-h light/dark cycle. Mice were given water and LabDiet 5010 chow (PMI Nutrition International) ad libitum. This research was approved by the CWRU Institutional Animal Care and Use Committee.

**Sox9Ods crosses.** 129T1/SvJ- 

**Genotyping.** All N2 mice were genotyped to validate phenotypes. The following PCR primers were used: OdsF, cgattttgctcagcaagcct; OdsR, cctatggaatcatgcgcgtc. SRY was digested with 5 units of Ddel (New England Biolabs) for at least 2 h at 37°C before electrophoresis in 4% UltraPure agarose (Invitrogen) in 1× TAE. The results were analyzed using the χ² goodness-of-fit test. The significance threshold for all calculations was set at P < 0.05.

**Histology and immunohistochemistry.** Gonads were isolated from neonatal pups within 24 h of birth (P1) and from adults (P21-P35), fixed overnight at 4°C in formalin, rinsed once in 1× PBS at room temperature, and equilibrated in 30% sucrose in 1× PBS for at least 2 d before embedding and freezing in OCT compound (Sakura Finetek USA). Embedded gonads were sectioned at 5 to 10 μ with a Leica CM3650 cryostat and sections were dried in the dark at room temperature for 1 h. For histology, sections were stained with H&E. For immunohistochemistry, IHC-Fr was conducted according to the manufacturer's recommended protocol. Rabbit polyclonal Mvh primary antibody (Abcam) was diluted 1:100. Goat polyclonal to rabbit IgG secondary antibody (Abcam) was diluted 1:100. An antigen retrieval step was not necessary. Substrate development was performed with the Vector VIP Substrate kit (Vector Laboratories). Developed slides were dehydrated in an ascending ethanol series, cleared in xylene, and mounted with VectaMount permanent mounting medium (Vector Laboratories).

Results

The 129/Sv Y chromosome is not necessary for TGCTs. To test whether the Y chromosome from the susceptible strain has genetic variants that are necessary for TGCTs, we created two new CSSs that produce 129/Sv males with substituted Y chromosomes derived from the TGCT-resistant MOLF/Eij or C57BL/6j strains (129-Chr Y^{MOLF/Eij} and 129-Chr Y^{B6}, respectively). We then screened males of these CSSs for TGCTs. We expected that if the Y chromosome derived from 129/Sv had genetic variants that are necessary for TGCTs, no TGCTs would be found in CSS males with alternative Y chromosomes. We report the number and percentage of control (129/Sv) and test (CSS) males affected with at least one TGCT (Table 1). The percentage of affected control 129/Sv males, 4.8%, agrees with published estimates for 129/Sv (21, 29). Among a total of 291 surveyed males in the two CSSs, the frequency of affected males (3.8%) agreed closely with the percentage of affected 129/Sv control males (129-Chr Y^{B6} versus 129/Sv; P = 0.97; 129-Chr Y^{MOLF/Eij} versus 129/Sv; P = 0.24), showing that the 129/Sv Y chromosome does not carry genetic variants that are necessary for TGCTs.

The 129/Sv Y chromosome is not sufficient for TGCTs. To test whether the 129/Sv Y chromosome has genes that are sufficient for TGCTs, we made a B6-Chr Y^{129} CSS that produces males from the TGCT-resistant C57BL/6j strain but with Y chromosomes derived from the TGCT-susceptible 129/Sv strain. We surveyed the CSS for TGCTs, expecting that if the 129/Sv Y chromosome has genetic variants that are sufficient for TGCTs, we would find affected B6-Chr Y^{129} males (Table 1). With a single exception, affected males were not found among the 391 males that were surveyed. Regrettably, the sample for the putative TGCT was lost before histologic validation. The exception may represent the action of a weak sufficiency gene or a rare case of TGCT outside the 129 inbred strains, or it may not be a TGCT. It would take thousands of mice to differentiate between these alternative hypotheses. Nonetheless, this survey rules out a genetic variant on the 129/Sv Y chromosome that acts as a strong sufficiency factor.

**Table 1. TGCTs in CSS males with alternate Y chromosomes**

<table>
<thead>
<tr>
<th>Inbred strain</th>
<th>n examined</th>
<th>% affected with TGCTs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control males</td>
<td>129/Sv</td>
<td>146</td>
</tr>
<tr>
<td>CSS males</td>
<td>129-Chr Y^{B6}</td>
<td>144</td>
</tr>
<tr>
<td>129-Chr Y^{MOLF}</td>
<td>147</td>
<td>2.7</td>
</tr>
<tr>
<td>B6-Chr Y^{129}</td>
<td>391</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**NOTE:** The single putative TGCT in the B6-Chr Y^{129} CSS was lost before it could be verified histologically.


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breeding strategy that minimizes the number of backcrosses to 129/Sv but increases the number of males with a TGCT by introducing two genetic variants, namely Dnd1Ter and MOLF-19, which enhance susceptibility on the 129/Sv background (Fig. 1; refs. 32, 33).

In Table 2, we report the percentage of sex-reversed (test) and wild-type (control) N2 males that were affected with at least one TGCT. All sex-reversed N2 males were either XX MOLF-19/+ Sox9Ods/+ Dnd1Ter/+ or XX MOLF-19/+ Sox9Ods/+ Dnd1+/+. We also present data from two of the four genotypes of normal N2 males in Table 2 (see Supplementary Table S1 for TGCT data from all genotypes). Because only 13% of the N2 progeny were sex reversed, large numbers of N2s needed to be screened. Interestingly, neither Dnd1Ter/+ nor Dnd1+/+ sex-reversed males had TGCTs, whereas both Dnd1Ter/+ and Dnd1+/+ control males developed TGCTs ($P < 3.9 \times 10^{-5}$), suggesting that TGCTs do not occur in the absence of the Y chromosome.

The testes of sex-reversed mice were approximately one third the size of normal testes (Fig. 2), so we considered the possibility that TGCTs might also be small. To ensure that small TGCTs were not overlooked, we examined 20 serially sectioned sex-reversed testes histologically, but tumor foci were not found. Although several cases of ovotestes, with both ovarian and testicular tissue in the same gonad, were observed in sex-reversed males, most gonads appeared as normal testes.

We considered the possibility that PGCs were lost before TGCTs could form in sex-reversed males. TGCTs in mice initiate from PGCs during a narrow developmental window, between E11.5 and E13.5 (34). Embryos that do not develop a TGCT during this time period will not have testis tumors as adults. To test for the presence of PGCs, we used an antibody against a PGC marker, mouse vasa homologue (Mvh; refs. 35, 36). We confirmed that sex-reversed XX males had PGCs at birth (Fig. 2), albeit fewer than their normal XY littermates (Fig. 2), and that they were germ cell deficient as adults (Fig. 2), as is the case in sex-reversed XX FVB/NJ males (27). Interestingly, germ cell deficiency is a predisposing condition for TGCTs, with mutant genes such as KitlSl and Dnd1Ter showing both reduced PGC numbers and an increased frequency of affected males (37, 38).

We expected that if TGCTs form in sex-reversed embryos, they should persist and be macroscopically evident as adults. Because TGCTs were absent in sex-reversed adults, we considered the possibility that TGCT might form but not progress owing to a potentially abnormal hormonal or gonadal environment (e.g., ovotestes were observed in several sex-reversed XX males). To test this hypothesis, we serially sectioned the testes of 12 XY Dnd1Ter/+ males, 2 XY Dnd1Ter/Ter males, and 23 XX sex-reversed males at birth and looked microscopically for tumor foci. TGCT foci were not found in testes from sex-reversed males, whereas we identified one affected testis in the XY Dnd1Ter/+ males and one affected testis in the XY Dnd1Ter/Ter males. Together, these results show that PGCs are present during the critical developmental period of tumorigenesis and that sex-reversed mice do not have TGCTs or, if they do, they have formed and regressed before birth.

**Table 2. Test for TGCTs in control and sex-reversed males**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype</th>
<th>n examined</th>
<th>% affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control males</td>
<td>XY Sox9Ods+/− Dnd1Ter/+</td>
<td>146</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>XY Sox9Ods+/− Dnd1+/−</td>
<td>127</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>273</td>
<td></td>
</tr>
<tr>
<td>Sex-reversed males</td>
<td>XX Sox9Ods+/− Dnd1Ter/+</td>
<td>81</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>XX Sox9Ods+/− Dnd1+/−</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>137</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Here, we show the number of adult N2 males examined and percentage affected with TGCTs. All males are obligate MOLF-19+/+ heterosomics.
Discussion

TGCTs account for 1% of all male cancers and have an unusually strong genetic component (1–3). To date, research into the role of Y-linked factors in TGCT etiology has produced evidence for TGCT-promoting and TGCT-suppressing genes on the human Y chromosome (5, 6, 16–19). By surveying a total of 1,704 males from several populations of genetically engineered mice, we sought to clarify the role of the Y chromosome on TGCT susceptibility in an established mouse model. In doing so, we found that the mouse Y chromosome has at least one genetic factor that is necessary for TGCTs.

We tested two hypotheses: the first involved testing whether alternative Y chromosomes have genetic variants that affect susceptibility, and the second tested whether the presence of a Y chromosome is required for tumorigenesis. By using CSSs with alternative Y chromosomes, we tested whether the Y chromosome from the susceptible 129/Sv strain is necessary for TGCTs. 129/Sv males with Y chromosomes derived from the C57BL/6J and MOLF/EiJ inbred strains developed TGCTs at the expected frequencies (Table 1), showing that the 129/Sv Y chromosome does not have unique or strong genetic variants that are necessary for TGCTs. This test also involved determining whether the Y chromosome from the 129/Sv strain has genetic variants that are sufficient for TGCTs. Absence of several affected B6-Chr Y129 males suggests that a strong 129-derived sufficiency factor is not involved.

These CSS-based tests depend on the extent of genetic variation between the tested Y chromosomes. Various evidence shows that Y chromosomes in most inbred strains of mice, including 129/Sv, C57BL/6J, and MOLF/EiJ, are derived from Mus musculus molossinus (39, 40). However, two of the inbred strains (129/Sv and C57BL/6J) were derived from wild mice more than 100 years ago, whereas MOLF/EiJ was independently derived within the last 40 years (41, 42). The Y chromosome in the MOLF/EiJ strain should therefore show comparable levels of genetic variation with other outbred or recently inbred molossinus Y chromosomes, whereas the molossinus-derived Y chromosomes among traditional inbred strains should show only mutational divergence that arose since these inbred strains were established. Unfortunately, the Y chromosome has proven difficult to sequence (43), a consequence of its highly repetitive nature. Consequently, it is not yet possible to rigorously evaluate sequence divergence among these Y chromosomes. However, spontaneous mutants with functional effects between the Y chromosomes of related inbred strains have been reported. Using a Y chromosome CSS, Maxson and colleagues (44) showed the DBA/1Bg Y chromosome has spontaneous mutants that promote male aggression, which the C57BL/10 Y chromosome lacks, despite both having a Mus musculus molossinus–derived Y chromosome (39, 40). Together, these results and observations strongly suggest that spontaneous Y chromosome mutants on the 129/Sv Y chromosome do not account for the genetic susceptibility to TGCTs in 129/Sv.

The second hypothesis that we tested involved determining whether any Y chromosome was necessary for TGCTs by using a sex-reversing mutant that produces XX males with testes but lacking Y chromosomes. XY but not XX sex-reversed males had TGCTs (Table 2), showing that at least one Y-linked factor is necessary for development of TGCTs. By using crosses that included segregating controls, we were able to control for maternal and genetic background effects. However, we did not test whether the hormonal profiles of sex-reversed mice appropriately mimic that of normal males, and a few cases of oovestes were found, raising the possibility that the hormonal profile in sex-reversed males is not fully compatible with normal gonadal function.

Evidence from several TGCT modifier genes indicates that germ cell deficiency is correlated with increased rates of TGCT. For example, XY Dnd1Ter/Ter testes are devoid of germ cells at birth, but 94% of adult males present with a TGCT (38). However, not all forms of germ cell deficiency are associated with increased risk for TGCTs. For example, KitW mutants are germ cell deficient (45) but develop TGCTs at rates comparable with 129/Sv males (32, 37). Therefore, sex-reversed testes are severely germ cell deficient (compare normal versus sex-reversed; Fig. 2A), sex-reversed males

Figure 2. Comparison of testes from normal males (XY Sox9OdsOds Dnd1TerTer) and sex-reversed males (XX Sox9OdsOds Dnd1TerTer). A, germ cells (purple) were present at birth in both normal and sex-reversed testes. Secondary antibody failed to detect germ cells in the absence of primary anti-Mvh antibody in both normal and sex-reversed testes. B, H&E-stained sections through 5-wk-old adult testes with and without TGCTs. Normal male testes contained seminiferous tubules with an abundance of germ cells (arrow), whereas adult sex-reversed testes contained Sertoli cells but were devoid of germ cells. A TGCT from a sex-reversed male was sectioned, revealing muscle, adipose, and cartilage. In contrast, no TGCTs were found in adult sex-reversed gonads. C, morphology of normal male testes, TGCTs, and sex-reversed testes. From left to right, a pair of morphologically normal testes, a case of bilateral TGCT, a case of unilateral TGCT with unaffected testis, and two pairs of sex-reversed testes. Note that sex-reversed testes are approximately two thirds smaller than normal male testes but are otherwise morphologically normal.
could show increased risk of TGCT. However, sex-reversed males lack TGCTs, strongly implicating Y chromosome factors in TGCT development. Perhaps presence of two X chromosomes, rather than loss of the Y chromosome, accounts for absence of TGCTs in sex-reversed mice. However, this scenario is unlikely because relatively few genes escape X inactivation and more importantly because 47 XXY individuals with Klinefelter’s syndrome have high rates of germ cell tumors, despite having two X chromosomes (46, 47). Our results therefore strongly support the hypothesis that a Y chromosome factor that does not differ between 129/Sv, C57BL/6j, and MOLF/ Eij is required for TGCTs.

In our study, sex-reversed XX males, which lacked all Y-derived sequences, failed to develop TGCTs at both time points tested. In a separate study, Cook and colleagues5 showed that TGCT foci were evident at E18.5 in XX Dnd1/Ter/Term testes of sex-reversed males resulting from the action of an autosomal copy of Sry, Sry,ATC. These results suggest that Sry may be the gene on the Y chromosome that is required for TGCTs. This in turn implies that Sry has another function in the embryonic gonad, aside from activation of Sox9 and male sex determination. Taken together with the data of Cook and colleagues, our results strongly raise the possibility of a novel role for Sry in the development of TGCTs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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18. Heimdal K, Olsson H, Tretli S, Flodgren P, Børresen A, Nelson SO, et al. Sequence of the mouse Y chromosome that is required for TGCTs. This in turn implies that Sry, Sry,ATC. These results suggest that Sry may be the gene on the Y chromosome that is required for TGCTs. This in turn implies that Sry has another function in the embryonic gonad, aside from activation of Sox9 and male sex determination. Taken together with the data of Cook and colleagues, our results strongly raise the possibility of a novel role for Sry in the development of TGCTs.

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