Enhancers and Suppressors of Testicular Cancer Susceptibility in Single- and Double-Mutant Mice

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ABSTRACT

Susceptibility to spontaneous testicular germ cell tumors (TGCTs), a common cancer affecting young men, shows unusual genetic complexity. Despite remarkable progress in the genetics analysis of susceptibility to many cancers, TGCT susceptibility genes have not yet been identified. Various mutations that are inherited as Mendelian traits in laboratory mice affect susceptibility to spontaneous TGCTs on the 129/Sv inbred genetic background. We compared the frequency of spontaneous TGCTs in single- and double-mutant mice to identify combinations that show evidence of enhancer or suppressor effects. The lower-than-expected TGCT frequencies in mice with partial deficiencies of TRP53 and MGF-SLJ and in 129.MOLF-Chr19 (M19) consomic mice that were heterozygous for the Ay mutation suggest that either these genes complement each other to restore normal functionality in TGCT stem cells or together these genes activate mechanisms that suppress incipient TGCTs. By contrast, the higher-than-expected TGCT frequencies in MgfSl-J – M19 compound heterozygous mice suggest that these mutations exacerbate each other’s effects. Together, these results provide clues to the genetic and molecular basis for susceptibility to TGCTs in mice and perhaps in humans.

TESTICULAR germ cell tumors (TGCTs) are the most common cancer affecting young men. The incidence of TGCTs has doubled within the last 50 years, predominately in Eastern Europe, indicating that environmental factors affect susceptibility (Buetow 1995). Genetics also contributes to TGCT susceptibility, with an increased risk of 8- to 10-fold among brothers and 4-fold among sons of affected individuals (Forman et al. 1992; Lindelof and Eklund 2001). Despite its prevalence, little is known about the genetic control of TGCT susceptibility. Mapping studies have revealed many weak autosomal linkages (Leahy et al. 1995; Bishop 1998) but only one significant linkage at Xq27 (Rapley et al. 2000). To date, TGCT susceptibility genes have not yet been identified in humans. Linkage studies have been difficult in part because of the limited number of multigenational pedigrees with sufficient numbers of affected individuals. The 129/Sv inbred strain of laboratory mice is an important model for unraveling the complexity of the genetic control of susceptibility and resistance to TGCTs (Stevens and Hummel 1957). Linkages and genes discovered in studies of these mice could be used to guide studies in humans.

TGCTs are rare in most inbred mouse strains and occur spontaneously at a rate of 1–5% in the 129/Sv inbred strains (Stevens and Hummel 1957). Various characteristics of TGCTs in these strains suggest that 129/Sv mice are an important model for pediatric germ cell tumors that occur in humans (Stevens 1967; Jiang and Nadeau 2001; Lam and Nadeau 2003). These tumors originate during fetal development and are composed of various cell types and tissues at different stages of differentiation. In both humans and mice, males develop pediatric germ cell tumors before puberty and these tumors are classified histologically as nonseminomas.

In mice, primordial germ cells (PGCs) are the earliest recognizable precursors of gametes and arise outside the gonads (Molyneaux et al. 2001). During embryogenesis, PGCs are first identified around embryonic day 7 (E7) at the primitive streak. The PGCs leave the yolk sac, migrate through the dorsal mesentery of the gut, and arrive at the genital ridges by E11.5. As the PGCs migrate toward the genital ridges, they proliferate, with the number increasing from ~40 at E7 to ~2000 at E11.5 when they arrive at the genital ridges. At E13.5, PGCs enter G1 mitotic arrest and remain arrested until a few days after birth (Donovan et al. 1998). This period from E11.5 to E13.5 is thought to be when TGCT development begins. It has been hypothesized that PGCs fail to enter G1 mitotic arrest and continue to divide into pluripotent stem cells called embryonal carcinoma cells that in turn become tumors composed of various cells and tissue types (Jiang and Nadeau 2001).

Several mutations that are inherited as Mendelian traits affect TGCT susceptibility in 129/Sv mice. These mutations must be congenic on the 129/Sv inbred ge-
netic background to exert their influence, demonstrating that these mutant genes act together with 129/Sv-derived genes to control susceptibility.

The Steel (Sl) and White-spotting (W) mutants were first identified as coat color variants (Silver 1995). Sl mutants result from molecular lesions in the mast cell growth factor (Mgf) gene (Copleand et al. 1990) and W mutants result from lesions in the kit oncogene, which encodes the c-kit tyrosine kinase cell surface receptor for the MGF ligand (Charbot et al. 1988). Mice with mutations in these genes are defective in melanogenesis, gametogenesis, and hematopoiesis. The MGF-KIT signaling pathway is required for PGC survival and proliferation during germline development (Dolci et al. 1991).

Stevens transferred several Sl and W mutations onto the 129/Sv inbred strain to study tumorigenesis and found that only Sl and Sl/+ alleles cause a significant increase in the frequency of TGCTs, whereas other alleles of Sl and W mutations did not affect susceptibility (L. C. Stevens, personal communication). Mgf Sl is a spontaneous mutation that results from an ~640-kb deletion that includes the Mgf gene (Bedell et al. 1996). Homozygous Mgf Sl+/Mgf Sl/+ mice are PGC deficient and embryonic lethal (Stevens 1967). Heterozygous 129/Sv-Mgf Sl/+ males have a tumor incidence more than double (12–14%) the rate in their wild-type controls (5%, Stevens 1967).

Trip53 is a tumor suppressor gene that arrests cells to allow proper repair of DNA damage (Kastan et al. 1991). To study the role of TRP53 in tumorigenesis, a null mutation was created by homologous recombination in mouse embryonic stem (ES) cells (Donehower et al. 1992; Jacks et al. 1994). Mice homozygous for the null allele appear normal but are prone to a variety of tumors, with lymphomas being the most common (Donehower et al. 1992; Jacks et al. 1994). The spectrum of tumors varies among TRP53-deficient mice on different genetic backgrounds (Donehower et al. 1995). Loss of TRP53 appears to accelerate existing tumor predisposition in a background-dependent manner. In a mixed C57BL/6 and 129/Sv genetic background, only 9% of the TRP53-deficient mice develop testicular tumors, whereas on a pure 129/Sv genetic background, 35% of the TRP53-deficient mice develop testicular tumors (Donehower et al. 1995).

A genome scan suggested that at least one gene on chromosome 19 of the MOLF/Ei inbred strain dramatically increases susceptibility to TGCTs (Collin et al. 1996). 129.MOLF-Chr19 is a chromosome substitution strain (CSS), also known as a consomic strain, in which chromosome (Chr) 19 of 129/Sv is replaced by MOLF-derived chromosome 19 on the 129/Sv background (Matin et al. 1999). Homosomic mice, in which both copies of chromosome 19 are derived from MOLF/Ei, have a tumor incidence of ~80%: ~50% of these males have bilateral tumors as compared to the TGCT incidence of 5% with most cases being unilateral in 129/Sv males (Matin et al. 1999). Heterosomic animals, which have only one copy of the MOLF-derived chromosome 19, have a tumor incidence of 20%, and only 2–3% of the affected animals have bilateral tumors (Matin et al. 1999; Youngren et al. 2003).

A' is a mutation with dominant effects at the Agouti (a) locus on mouse chromosome 2 (Michaud et al. 1994). The A' mutation results from an ~170-kb deletion of the entire coding region of the Raly gene, which is proximal to the Agouti gene. This deletion places the coding region of Agouti under the transcriptional control of the Raly promoter, resulting in ubiquitous expression of the agouti gene product (Michaud et al. 1993, 1994; Khrebtukova et al. 1999). The A’ phenotype is associated with obesity, diabetes, and yellow coat color (Bultman et al. 1992). A' mice also show increased susceptibility to pulmonary and mammary gland tumors in females, induced skin tumors in both sexes, and hepatomas in males (Stevens 1967). Remarkably, 129/Sv mice carrying the A’ mutation have approximately one-tenth the incidence of testicular germ cell tumors as the wild-type littermates (Stevens 1967).

Testicular cancer is a complex trait that may require as many as six independently segregating genes with additive and recessive effects (Stevens 1967). The low frequency of spontaneous TGCTs in the 129/Sv inbred strain and the multigenic basis of the cancer together make it difficult to dissect the genetic control of susceptibility of TGCTs. We used single gene mutations that are inherited as Mendelian traits to gain clues to the nature of genes and pathways that affect TGCT susceptibility. The various mutations on the 129 genetic background increase or decrease penetrance perhaps by activating novel pathways involved in tumorigenesis. The interaction of these Mendelian traits provides a unique opportunity to study TGCT development. In other model systems, interactions between different pairs of genes have provided unique insights into developmental pathways and cancer susceptibility (Moore et al. 1990; Oca Luna et al. 1995; Lamoreux et al. 2001; Montes de Parant and Lozano 2003). We therefore tested interactions between pairs of TGCT susceptibility genes to evaluate their effect on tumor frequencies, laterality of the tumors, and parental effects to reveal more about the molecular and developmental pathways leading to tumorigenesis.

**MATERIALS AND METHODS**

**Mice:** 129S1/SvImJ (JR002448, previously known as 129/SvJ and 129S3/SvImJ), 129S1/Sv-^*y*^*a*/*y*^*a^ (JR000090), B6.Cg-^*A*^/^*Ay*^ (JR000021), and 129-^*Trip53*^/^*Trp53*^ (JR002080) were obtained from the Jackson Laboratory (Bar Harbor, ME). The nomenclature for 129 substrains has been revised by the Jackson Laboratory (http://www.informatics.jax.org/rgchome/nomen/strain_129.shtml) and the recommended designations were used in this article. The 129.MOLF-Chr19 CSS (N15F2+) was described previously (Matin et al. 1999) and
was obtained from our research colony. Mice were maintained in the Case Western Reserve University Animal Resource Center on a 12-hr:12-hr light:dark cycle and fed Lab Diet 5010.

**Construction of a 129-A^r congeneric strain:** To transfer the A^r mutation onto the 129/Sv background, C57BL/6J-A^r mice were crossed to 129S1/SvImJ for 11 generations. Genes in the 129 background are essential for tumorigenesis, and therefore to perform the interaction tests, A^r must be transferred to and tested on the 129/Sv background.

**Construction of 129-A^r/+ , MOLF-Chr 19 congeneric congenic mice:** 129-A^r mice were crossed to our 129MOLF-Chr 19 CSS. Heterosomic 129MOLF-Chr 19 mice carrying A^r were then backcrossed to 129.MOLF-Chr 19. A^r progeny from these backcrosses were typed for simple sequence length polymorphisms along the length of chromosome 19. A^r progeny that were homosomic for chromosome 19 were selected to establish the test colony and wild-type littersmates were used as controls.

**Genotyping:** DNA for PCR genotyping was obtained from samples of tail tissue. Each tail sample was digested in 80 μl of water, 10 μl 10× PCR buffer, and 1 μl protease K (10 mg/ml).

**Trp53:** In this study, we purchased the Trp53/+ mutant strain developed by Jacks et al. (1994) from the Jackson Laboratory. This strain has a Trp53 mutant allele different from that reported by Donehower et al. (1992). In both strains, Trp53 heterozygous mutants rarely develop TGCTs, whereas they are more common in Trp53 homozygous mutants. TGCT frequencies of the heterozygous or the homozygous Trp53 mutants were not reported by Jacks et al. (1994), whereas Harvey et al. (1993) reported that their homozygous mutants developed TGCT at 35% and that the heterozygous mutants were reported to rarely develop TGCTs.

A three-primer PCR assay was used to distinguish wild-type from heterozygous Trp53 animals. The three primers are X7, 5’-TATATCTACAGAGCCGCTT-3’; Neo19, 5’-CATATCAAGGACAC-3’; and X6.5, 5’-GACGCGTGGTGACCTAT-3’. Primers X7 and Neo19 amplify a 600-bp fragment identifying the Neo insert. Primers X7 and X6.5 amplify a 400-bp fragment from the untargeted Trp53 allele. PCR amplification was carried out in a 96-well block MJ Research (Watertown, MA) PTC-200 thermal cycler. The reagents were 0.15 μl (0.75 units) Tag polymerase (Invitrogen, San Diego), 2.5 μl 10× PCR buffer (magnesium free), 0.5 μl 10 mM dNTPs, 1 μl 25 mM magnesium chloride, 0.2 μl of each primer (0.1 μM), 1 μl DNA (25 ng), and 19.35 μl H2O in a final volume of 25 μl. PCR conditions were as follows: initial denaturation step for 94° for 2 min followed by 35 cycles of 94° for 1 min, 60° for 1 min, 72° for 1 min, final extension of 72° for 5 min, and then 4° for 15 min. PCR products were resolved on a 2% agarose gel and visualized with ethidium bromide.

**Mgf^Sl/J:** The breakpoints of the Mgf^Sl/J deletion are not known and as a result a PCR genotyping assay is not available for the Mgf^Sl/J mutation. Mgf^Sl/J/+ mutant heterozygotes have a light coat color in the belly and the tips of the tail and digits are pink.

**RESULTS**

**Control tests—TGCT frequencies in single-mutant mice:** We used the frequency of TGCTs in single-mutant mice to calculate the expected frequency in double-mutant mice. If mutant genes have functionally independent and additive effects on tumorigenesis, the expected tumor frequency in double-mutant mice should be the sum of the frequencies in the relevant single-mutant mice. Rather than simply using TGCT frequencies reported in the literature, we measured the frequencies in our colony (Table 1). We also characterized the laterality of the tumors and tested for parental effects by comparing the tumor frequencies in reciprocal crosses that were not previously tested.

**Mgf^Sl/J, Mgf^Sl/J/+ heterozygotes typically have a TGCT frequency of 12–14%, on the basis of surveys of exceptionally large numbers of mice (Stevens 1967). We found that Mgf^Sl/J/+ heterozygotes had similar frequencies of TGCTs in reciprocal crosses (χ^2 = 0.13, P > 0.05), suggesting that parental factors did not affect susceptibility. The TGCT frequency in the pooled data of 170 Mgf^Sl/J/+ heterozygotes was 12% (Table 1A).

**Trp53:** The rate of affected Trp53/+ heterozygotes has not been reported (Donehower 1995). We found that Trp53/+ heterozygotes had similar TGCT frequencies in reciprocal crosses (χ^2 = 0.17, P > 0.5). In the interaction crosses between Trp53/+ and Mgf^Sl/J/+ heterozygous mutants, the single mutant Trp53/+ littermates pooled from reciprocal crosses had a tumor frequency of 15% (Table 5).

**129MOLF-Chr19 (M19):** The tumor frequency of 129MOLF-Chr19 was reported to be 82% with equal frequencies of unilateral and bilateral tumors (Matin et al. 1999). 129MOLF-Chr19 mice were backcrossed to 129/Sv mice to obtain heterosomic animals (only one copy of MOLF-Chr19). We found that heterosomic mice had similar frequencies of TGCTs in reciprocal crosses (χ^2 = 0.88; P > 0.1). The tumor frequency in the pooled sample of 300 heterosomic animals was 33% and tumors were mainly unilateral (29%; Table 1B).

**A^r:** The tumor frequency of 129/Sv mice carrying the A^r mutation was first reported by Stevens (1967) to be ~1%, whereas their wild-type littermates had a frequency of ~8% (χ^2 = 24.4, P<0.001). To verify Stevens’ discovery, we made a 129/Sv-A^r congenic strain and then transferred the A^r mutation onto the 129MOLF-Chr19 CSS background. This CSS strain has a tumor frequency of ~80% and therefore serves as a statistically powerful way to quickly determine whether A^r suppresses tumori-
termates had a TGCT frequency of 32%, which was not mutants of parental crosses between C2 females laterality, and parental effects in the double mutants. tween the reciprocal A2/H11001 were used to test for interactions between TGCT susceptibility (Table 2). There was no evidence unilateral (Table 3A).

Double-mutant interaction tests: Double-mutant mice were used to test for interactions between TGCT susceptibility genes. We evaluated tumor frequency, tumor laterality, and parental effects in the double mutants. 

Mgf Sl-J and M19 double heterozygote: The double-heterozygous mutants of the interaction test between Mgf Sl-J/+ and M19 revealed a higher-than-expected TGCT susceptibility (Table 2). There was no evidence of parental effects between the reciprocal crosses (χ² = 0.83; P > 0.1); the data for the two crosses were therefore the interaction cross between C2/H11001 and wild-type littermates of C2 males, 20% of 89 double-heterozygous mutants developed TGCTs, of which only 2% were bilateral tumors (Table 3B). In the reciprocal cross of Mgf Sl-J/+ females × C2 males, 20% of 89 double-heterozygous mutants developed TGCTs, of which only 3% were bilateral tumors (Table 3C). Double-heterozygous mutants from both congenic crosses had low tumor frequencies and, importantly, rarely developed bilateral tumors. It appears that Mgf Sl-J did not interact separately

### TABLE 1
Control tests

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sample size</th>
<th>% unilateral tumors</th>
<th>% bilateral tumors</th>
<th>% total affected animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Control test between 129/Sv and Sl/++: overall tumor frequency of Sl/++ and wild-type controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sl/++</td>
<td>170</td>
<td>11 (19)</td>
<td>1 (2)</td>
<td>12 (21)</td>
</tr>
<tr>
<td>+/+</td>
<td>191</td>
<td>8 (15)</td>
<td>0</td>
<td>8 (15)</td>
</tr>
<tr>
<td>B. Control test between M19 and 129/Sv: overall tumor frequency of heterosomic animals</td>
<td>Heterosomic</td>
<td>300</td>
<td>29 (86)</td>
<td>4 (13)</td>
</tr>
</tbody>
</table>

### TABLE 2
Interaction test between M19 and Mgf Sl-J in heterozygous mutants: overall tumor frequency of M19/+ Sl/++ and wild-type controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sample size</th>
<th>% unilateral tumors</th>
<th>% bilateral tumors</th>
<th>% total affected animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>M19/+ , Sl/++</td>
<td>215</td>
<td>38 (82)</td>
<td>19 (40)</td>
<td>57 (122)</td>
</tr>
<tr>
<td>M19/+ , +/+</td>
<td>225</td>
<td>26 (58)</td>
<td>6 (14)</td>
<td>32 (72)</td>
</tr>
</tbody>
</table>
with either the proximal or the distal segments of the MOLF-Chr19 to increase the incidence of bilateral tumors.

**M19 homosomics and Mgf<sup>sl</sup> congenic heterozygotes:** Homosomic-congenic mutants were generated by transferring Mgf<sup>sl</sup> to the 129.MOLF-Chr19 strain. If M19 and Mgf<sup>sl</sup> acted additively, the expected tumor frequency in the M19/M19 Mgf<sup>sl</sup>/+ mice would be 94% (= 82% (M19/M19) + 12% (Mgf<sup>sl</sup>/+)). The TGCT frequency in the homosomic-congenic-heterozygous mutants of the reciprocal crosses was similar ($\chi^2 = 3.65; P > 0.05$). The homosomic-congenic mutant had the expected high tumor frequency with an affected rate in the pooled data of 92% (Table 4), which was not significantly different from the expected additive frequency of 94% ($\chi^2 = 0.4; P > 0.5$). The M19/M19 +/+ littermates had a TGCT frequency of 80%, which was not significantly different from the expected tumor frequency (Matin et al. 1999). Previously, we reported (Table 2) that the double-heterozygous mutants had a significant increase of bilateral tumors; this was not observed in the homosomic-congenic mutants.

**Trp53/+ and Mgf<sup>sl</sup>/+ double-mutant heterozygotes:** The interaction test between Trp53/+ and Mgf<sup>sl</sup>/+ mutants revealed surprising and remarkable results. The tumor frequencies in the reciprocal parental crosses were not significantly different ($\chi^2 = 0.5; P > 0.1$). The pooled data for 93 wild-type littermates showed a tumor frequency of 2%, whereas in 91 Trp53/+ single-mutant littermates the pooled tumor frequency was 15%. Of 87 Mgf<sup>sl</sup>/+ single-mutant littermates, 11% developed TGCTs. Thus, the wild-type and single-mutant littermates had the appropriate rates of affected males, as compared to the control data. These TGCT frequencies were used to calculate the expected additive TGCT frequency for the Trp53/+ Mgf<sup>sl</sup>/+ double-heterozygous mutants. If the two mutant genes acted additively, the expected additive tumor frequency would be 26% (= 15% (Trp53/+)) + 11% (Mgf<sup>sl</sup> /+)). In the pooled data of 75 double-heterozygous mutants, only 7% developed a TGCT (Table 5). The double-heterozygous mutants had a tumor frequency fourfold lower than expected ($\chi^2 = 13.6; P < 0.001$), which suggests that partial deficiency of the tumor suppressor gene Trp53 and heterozygosity for the Mgf<sup>sl</sup> deletion suppressed development of TGCTs.

**A<sup>+</sup> and M19:** 129.MOLF-Chr19 carrying the A<sup>+</sup> mutation had a reduced TGCT frequency compared to that of 129.MOLF-Chr19 wild-type littermates without A<sup>+</sup> (Table 6). No evidence for significant parental effects was observed among progeny from reciprocal crosses. A total of 104 male progeny of 129.MOLF-Chr19 carrying the A<sup>+</sup> mutation were examined and 40% developed TGCTs, in comparison to 65% of the 105 129.MOLF-

### TABLE 3

Localization of the region on MOLF-Chr19 that interacts with Mgf<sup>sl</sup>

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sample size</th>
<th>% unilateral tumors</th>
<th>% bilateral tumors</th>
<th>% total affected animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2/+, Sl/+</td>
<td>130</td>
<td>23 (30)</td>
<td>7 (9)</td>
<td>30 (39)</td>
</tr>
<tr>
<td>A2/+, +/+</td>
<td>163</td>
<td>15 (24)</td>
<td>1 (1)</td>
<td>15 (25)</td>
</tr>
<tr>
<td>C2/+, Sl/+</td>
<td>97</td>
<td>8 (8)</td>
<td>2 (2)</td>
<td>10 (10)</td>
</tr>
<tr>
<td>C2/+, +/+</td>
<td>84</td>
<td>13 (11)</td>
<td>0</td>
<td>13 (11)</td>
</tr>
<tr>
<td>C2/+, Sl/+</td>
<td>89</td>
<td>18 (16)</td>
<td>2 (2)</td>
<td>20 (18)</td>
</tr>
<tr>
<td>C2/+, +/+</td>
<td>71</td>
<td>8 (6)</td>
<td>0</td>
<td>8 (6)</td>
</tr>
</tbody>
</table>

### TABLE 4

Interaction test between M19 and Sl/+ in homozygous-congenic mutants: overall tumor frequency of M19/M19 Sl/+ and wild-type controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sample size</th>
<th>% unilateral tumors</th>
<th>% bilateral tumors</th>
<th>% total affected animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>M19/M19, Sl/+</td>
<td>39</td>
<td>33 (13)</td>
<td>59 (23)</td>
<td>92 (36)</td>
</tr>
<tr>
<td>M19/M19, +/+</td>
<td>50</td>
<td>36 (18)</td>
<td>46 (23)</td>
<td>82 (41)</td>
</tr>
</tbody>
</table>
TABLE 5

Interaction test between Trp53 and Mgf<sup>Sl-J</sup>: overall tumor frequency of Trp53/+<em>SlJ</em>/+ and littermate controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sample size</th>
<th>% unilateral tumors</th>
<th>% bilateral tumors</th>
<th>% total affected animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp53/+&lt;em&gt;, SlJ&lt;/em&gt;/+</td>
<td>75</td>
<td>7 (3)</td>
<td>0</td>
<td>7 (5)</td>
</tr>
<tr>
<td>Trp53/+&lt;em&gt;, SlJ&lt;/em&gt;/+</td>
<td>91</td>
<td>15 (14)</td>
<td>0</td>
<td>15 (14)</td>
</tr>
<tr>
<td>+/+&lt;em&gt;, SlJ&lt;/em&gt;/+</td>
<td>87</td>
<td>11 (10)</td>
<td>0</td>
<td>11 (10)</td>
</tr>
<tr>
<td>+/+&lt;em&gt;, SlJ&lt;/em&gt;/+</td>
<td>93</td>
<td>2 (2)</td>
<td>0</td>
<td>2 (2)</td>
</tr>
</tbody>
</table>

Chr19 without the A<sup>l</sup> mutation that developed TGCTs (χ<sup>2</sup> = 12.4, P < 0.001). These results show that A<sup>l</sup> not only suppresses TGCT frequency in the 129/Sv inbred strain (Stevens 1967) but also causes a 25% reduction of TGCT incidence in the 129.MOLF-Chr19 CSS.

DISCUSSION

The low incidence of spontaneous TGCTs in the 129/Sv inbred strain makes it difficult to study the genetic control of TGCT susceptibility in these mice. In this article, Mendelian mutations that are known to individually increase or decrease the frequency of TGCTs were used to test interactions as a way to learn more about the pathways that control TGCT susceptibility.

The pairwise gene interaction tests showed that several mutant genes enhanced or suppressed the development of TGCTs. The M19/+<em>SlJ</em>/+ compound heterozygotes had a 27% increase in TGCT frequency over the expected additive rate. This increase resulted primarily from an unexpectedly high frequency of bilateral tumors. In contrast, an interaction between <em>Mgf</em><sup>Sl-J</sup> and <em>Trp53</em> suppressed the development of TGCTs by about fourfold in double heterozygotes, lowering the affected rate from the expected 26% to 7%, which is similar to the background rate of the 129/Sv strain. The A<sup>l</sup> mutation also suppressed the TGCT frequency in the 129.MOLF-Chr19 CSS.

Traditionally, mutations that increase susceptibility to cancers have been studied to learn more about the mechanisms that promote tumorigenesis. In this study, we discovered two pairs of interacting genes that suppressed development of TGCTs and another pair of genes that enhanced susceptibility. These suppressors and enhancers may provide an important way to modulate the genetic and molecular pathways that lead to TGCTs in mice and perhaps in humans.

Enhancers of TGCT susceptibility: The <em>Mgf</em><sup>Sl-J</sup>/+<em>M19</em>/+ double-heterozygous mutants showed that <em>Mgf</em><sup>Sl-J</sup> and at least two genes on chromosome 19 (one proximal and one distal) interacted to significantly increase the incidence of TGCTs. The increased frequency over the expected additive tumor frequency results from an increased incidence of bilateral tumors. Congenic strains that dissect the MOLF-Chr19 into two regions, proximal and distal, were crossed to the <em>Mgf</em><sup>Sl-J</sup>/+<em>M19</em>/+ mutants. We were, however, unable to localize the region on MOLF-Chr19 that interacted with <em>Mgf</em><sup>Sl-J</sup> to increase susceptibility to bilateral tumors. Heterozygous-congenic mutants showed that neither the proximal nor the distal end of the MOLF-Chr19 alone increased the incidence of bilateral tumors. These data complement the analysis of the panel of congenic strains to characterize TGCT susceptibility genes in the 129.MOLF-Chr19 CSS.

TABLE 6

Interaction test between M19 and A<sup>l</sup>: overall tumor frequency of M19/M19 A<sup>l</sup>/+ and wild-type controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sample size</th>
<th>% unilateral tumors</th>
<th>% bilateral tumors</th>
<th>% total affected animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>M19/M19, A&lt;sup&gt;l&lt;/sup&gt;/+</td>
<td>104</td>
<td>32 (33)</td>
<td>9 (9)</td>
<td>40 (42)</td>
</tr>
<tr>
<td>M19/M19, +/+</td>
<td>105</td>
<td>40 (42)</td>
<td>26 (26)</td>
<td>65 (68)</td>
</tr>
</tbody>
</table>
eral tumors may therefore require at least three factors: Mgf<sup>SlJ</sup>, together with the proximal and distal portions of MOLF-Chr19. Interestingly, the homosomic-congenic mutants Mgf<sup>SlJ</sup>/+ M19/M19 did not have an increased susceptibility to bilateral tumors, suggesting that the interaction observed in the double heterozygous mutants is complex and perhaps involves a dosage effect.

**Suppressors of TGCT susceptibility**: The interaction crosses revealed two new models to suppress TGCT susceptibility. Traditionally, genes that promote tumorigenesis are identified, with the ultimate goal of suppressing or preventing tumor formation by studying the mechanisms of tumorigenesis. Tumor suppression in mice such as the Trp53<sup>+/</sup> Mgf<sup>SlJ</sup>+/+ and Ay/+ M19/+/+ double-mutant mice, with their significantly reduced TGCT frequencies, may reveal clues to the genetic and molecular basis for modulating TGCT susceptibility.

**Trp53 and Mgf<sup>SlJ</sup>:** Mutations in Trp53 and Mgf<sup>SlJ</sup> on the 129/Sv inbred genetic background each increased susceptibility to TGCTs. In the interaction cross between Trp53<sup>+/</sup> and Mgf<sup>SlJ</sup>+/+, the double mutants had a tumor frequency remarkably lower (about a fourfold decrease) than the expected rate resulting from the additive effects of the single mutants. These results suggest that interactions between apoptosis and MGF-KIT signals modulate susceptibility to testicular cancer.

Trp53 mediates apoptosis and cell cycle arrest at the G<sub>S</sub>/S stage (Lee et al. 1994), whereas the signaling pathway of the MGF-KIT prevents apoptosis and promotes cell division and growth of melanocytes, mast cells, and germ cells (Dolci et al. 1991). In a cell culture system, MGF inhibits Trp53-mediated apoptosis and differentiation, but not G<sub>S</sub>/S cell cycle arrest (Abrahamson et al. 1995). Most studies that examine the relationship between KIT/ MGF signaling and TRP53 involve cell culture systems (Abrahamson et al. 1995; Matsui et al. 2000), whereas Jordan et al. (1999) used an in vivo model system to study the effect of Trp53 on melanocytes, mast cells, and germ cells by taking advantage of the Kit<sup>wt</sup> mutant, which is homozygous viable.

Mouse mutants of the KIT receptor and its ligand, MGF, are defective in melanogenesis, gametogenesis, and hematopoiesis. Although the phenotypes of most of the Kit and Sl mutants are similar, Stevens (1967) found contrasting results for several Kit and Sl alleles on the 129/Sv inbred background: Sl alleles (Sl and Sl<sup>d</sup>) developed TGCTs more than twice as often as did the wild-type littermates, whereas none of the Kit mutants showed an increased susceptibility of TGCTs. In a separate study, the Kit<sup>wt</sup> mutant was crossed to Trp53-deficient mutants to study the role of Trp53 in regulating cell death in the absence of MGF-KIT signaling (Jordan et al. 1999). The double mutants that were deficient in TRP53 function did not increase the survival of melanocyte and mast cells, but showed instead an increased number of germ cells and restored fertility (Jordan et al. 1999). Normally, MGF-KIT signals are induced between E7.5 and E13 (Manova and Bachvarova 1991) to inhibit TRP53 function in PGC proliferation (Abrahamson et al. 1995). The Kit<sup>wt</sup> mutants lack MGF-KIT signals and therefore cannot inhibit TRP53, leading to apoptosis of PGC and sterility. Jordan et al. (1999) demonstrated that double mutants deficient in Trp53 and Kit rescued sterility defects because apoptosis of male germ cells is Trp53 dependent but apoptosis of melanocyte and mast cells is regulated by a Trp53-independent pathway. This experiment explains how fertility can be restored in mutants that lack the functions of KIT and MGF, but it does not readily explain the suppression of TGCT development in the double heterozygous mutants Mgf<sup>SlJ</sup>/+ Trp53+/+. PGCs enter G<sub>1</sub> mitotic arrest at E13.5 until after birth (Donovan et al. 1998). If PGCs fail to enter G<sub>1</sub> mitotic arrest, they may become pluripotent stem cells, resulting in tumors of various cell and tissue types. The double-heterozygous mutants have a partial deficiency of TRP53 to inhibit apoptosis and bypass G<sub>1</sub> cell cycle check points, which could result in uncontrolled cell growth. In addition, the double-heterozygous mutants have the Sl<sup>J</sup> deletion, which can involve loss of a tumor suppressor gene or MGF itself that results in an increase of TGCT frequency. Surprisingly, the partial deficiency of Trp53 interacts with the Mgf<sup>SlJ</sup> mutation to suppress the development of TGCTs.

Additional experiments are needed to learn more about the mechanisms that suppress the development of TGCTs, such as immunohistochemistry to determine the localization of Trp53 within the testis of single- and double-mutant mice, measuring the levels of Trp53, and determining whether the interactions altered downstream or upstream the Trp53-mediated apoptosis or DNA repair pathway. From in vitro experiments, it does not seem likely that the MGF-KIT signaling pathway interacts directly with Trp53 functions, because the expression of other Trp53-dependent and downstream genes such as bax, p21, and mdm2 are not affected by the absence of the Steel factor (Abrahamson et al. 1995).

A<sub>1</sub>: Previous studies showed that A<sub>1</sub> mice have a greatly increased susceptibility to many different types of tumors (Wolf 1996; Wolff 1987). However, 129/Sv male mice that are heterozygous for the A<sub>1</sub> mutation showed an 10-fold reduction in TGCTs (Stevens 1967). The mechanism for this TGCT suppression was not pursued and Steven’s congenic strain was lost many years ago.

To verify findings of Stevens (1967), we created a new 129-A<sub>1</sub> congenic strain. 129/Sv mice normally develop TGCTs at a rate of 1–5%; Stevens therefore needed to examine a large number of males to find the 10-fold reduction. To detect a stronger effect with a smaller number of mice, we crossed A<sub>1</sub> congenic mice to a strain that has a high TGCT incidence, 129.MOLF-Chr19 (Matin et al. 1999). The M19/M19 strain of mice has a TGCT incidence of 80% (Matin et al. 1999) and therefore serves as an excellent strain to test for TGCT suppression.
suppression with $A'$. We found that the $A'$ mutation does indeed suppress TGCT formation and could serve a key role in understanding TGCTs and tumorigenesis in general.

Although the suppressive effect of $A'$ on TGCTs is exciting, little is known about the ways in which this mutation affects tumorigenesis. Because the $A'$ phenotype results from a large deletion upstream of agouti, two explanations for suppression are possible. The first is that the ubiquitous overexpression of agouti, because of the $A'$ mutation, results in TGCT suppression as a consequence of this unique expression pattern involving the melanocortin receptor signaling pathway. Another explanation for suppression is the deletion of $Raly$ or other genes between $Raly$ and agouti found in the 170-kb region upstream of agouti. The first model suggests that TGCT suppression may be linked to the ubiquitous expression of agouti. Agouti is normally expressed during development, in neonatal skin, and in the testis (Yen et al. 1994) and is responsible for the wild-type coat color seen in mice (Silvers 1979). Agouti expression results in the switching of eumelanin to phaeomelanin production by inhibition of $\alpha$-melanocyte-stimulating hormone due to antagonism of melanocortin receptor 1 (Bultman et al. 1992; Lu et al. 1994; Cone et al. 1996). However, in $A'$ mutants, ectopic agouti expression interferes with this switching pattern by preventing eumelanin production, leaving only phaeomelanin production. This results in the uniform yellow coat associated with the mutation (Cone et al. 1996).

When expressed ubiquitously, it is possible that the agouti protein interacts with other receptors not previously characterized. Characterization of the agouti gene revealed that multiple transcripts of varying sizes were expressed in the testis but not in any other adult tissues sampled. None of the testis-specific transcripts were expressed in neonatal skin or during development and they therefore play an undetermined role independent of pigmentation and development (Bultman et al. 1992). In $A'$ mutants a unique transcript is ectopically expressed in all tissues examined (Michaud et al. 1993). It is possible that in the $A'$ mutant the ubiquitous overexpression of this larger agouti transcript found in all adult tissues interacts with the testis-specific transcripts, leading to a suppression of TGCTs.

The second model suggests that TGCT suppression results from loss-of-function for genes deleted in the 170-kb region upstream of agouti. This deletion causes the coding region of agouti to be under the transcriptional control of the $Raly$ promoter, resulting in ubiquitous expression of the agouti gene product (Michaud et al. 1993). It is possible that deletion of the $Raly$ coding exons or other genes within this region suppresses TGCTs. $Raly$ is normally ubiquitously expressed and belongs to a family of RNA-binding proteins involved in pre-mRNA processing and embryonic developmental regulation (Michaud et al. 1993; Khrebtukova et al. 1999). $Raly$ functions in preimplantation development and its deletion accounts for the embryonic lethality associated with the $A'$ mutation (Duhl et al. 1994). Another gene located in the deletion between $Raly$'s promoter and agouti that could account for TGCT suppression is $\alpha$F2c2. $\alpha$F2c2 is a eukaryotic translation initiation factor involved in protein synthesis. Phosphorylation and dephosphorylation of $\alpha$F2c2 modulates protein translation (Lodish et al. 2000). Further studies involving engineered mutations are being pursued to identify the gene near $A'$ that suppresses TGCT susceptibility.

**Implications for TGCTs in humans:** Because of the prevalence of testicular cancer, an international effort is underway to identify susceptibility genes in humans (Bishop 1998). However, lack of multigenerational pedigrees with several affected individuals has made the search difficult. Family studies typically have limited power to detect linkage for complex traits; nevertheless, numerous weak linkages have been identified (Bishop 1998; Lindelof and Eklund 2001). This raises the possibility that additional linkages are yet to be discovered. Utilization of our mouse models provides an opportunity to control the genetics involved, thereby increasing the power of linkage studies. Approaches in the mouse and in humans will complement the search for genes involved in the initiation and progression of TGCTs.

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**LITERATURE CITED**


Collin, G. B., Y. Asada, D. S. Varnum and J. H. Nadeau, 1996 DNA pooling as a quick method for finding candidate linkages in multigenic trait results from a large deletion upstream of agouti, this deletion accounts for the embryonic lethality associated with the $A'$ mutation (Duhl et al. 1994). Another gene located in the deletion between $Raly$'s promoter and agouti that could account for TGCT suppression is $\alpha$F2c2. $\alpha$F2c2 is a eukaryotic translation initiation factor involved in protein synthesis. Phosphorylation and dephosphorylation of $\alpha$F2c2 modulates protein translation (Lodish et al. 2000). Further studies involving engineered mutations are being pursued to identify the gene near $A'$ that suppresses TGCT susceptibility.

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