Loss of the Transmembrane but not the Soluble Kit Ligand Isoform Increases Testicular Germ Cell Tumor Susceptibility in Mice

Jason D. Heaney, Man-Yee J. Lam, Megan V. Michelson, and Joseph H. Nadeau

Department of Genetics and Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, Ohio

Abstract

Several genetic variants act as modifiers of testicular germ cell tumor (TGCT) susceptibility in the 129/Sv mouse model of human pediatric TGCTs. One such modifier, the Steel locus, encodes the transmembrane-bound and soluble ligand of the kit receptor. Some (Sl and SIj) but not all (Sld) mutations of the Steel locus increase TGCT incidence in heterozygous mutant mice. Because Sl and SIj are large deletions that affect multiple transcripts and Sld is an intragenic deletion of the kit ligand (Kitl) from which only the soluble protein is produced, it was uncertain whether Kitl or a neighboring gene is a modifier of TGCT susceptibility. We tested the effect of the small Steel grizzle-belly (Slgb) deletion on TGCT susceptibility to determine whether Kitl is a TGCT modifier gene. An increase in TGCT incidence was observed in Slgb/+ heterozygotes, and fine mapping of the deletion breakpoints revealed that Kitl is the only conventional gene deleted by the mutation, suggesting that Kitl is the TGCT modifier gene at the Steel locus. Additionally, we propose that soluble Kitl in Sld/+ heterozygous mutant mice complements a dosage effect of transmembrane-associated kit ligand on TGCT susceptibility and that the kit receptor (Kit) is haplosufficient for primordial germ cell development.

Introduction

Testicular germ cell tumors (TGCT) are the most common cancer affecting young men (1). Although both environmental and genetic factors contribute to TGCT susceptibility, the genetic component of TGCTs is particularly strong. Statistically, 25% of susceptibility to early-onset TGCTs is attributable to genetic factors, making these tumors the third most heritable form of cancer (2). Despite the strong heritable influence on TGCT incidence and considerable effort to identify susceptibility genes, the genetic basis for TGCTs remains elusive. A recent study concluded that many loci with weak effects control TGCT susceptibility in heterozygotes (3–5). Variation among the Sl and W alleles was initially puzzling because it was difficult to understand how increased TGCT susceptibility could be limited to only two variants of this ligand-receptor complex. Discovery of the molecular nature of the Steel mutations provided clues. Sl and SIj are large deletions (~973 and ~650 kb, respectively) that remove all of Kitl and a significant amount of flanking sequence (Fig. 1A; refs. 18, 21). By contrast, Sld is a 4-kb intragenic deletion of Kitl that retains expression of soluble but not transmembrane Kitl at wild-type levels (Fig. 1A; refs. 22, 23). Therefore, two explanations are possible for increased TGCT incidence in Sl and SIj but not Sld mutant mice: (a) a gene or noncoding regulatory element neighboring Kitl is also deleted in Sl and SIj mutants and is responsible for increased TGCT susceptibility, or (b) gene dosage effects of soluble Kitl on TGCT susceptibility reduces TGCT incidence to 129/Sv levels in Sld mutants.

The Steel grizzle-belly (Slgb) mutation is a smaller deletion (~120 Kb) than Sl or SIj, with the 5’ breakpoint ~60 to 40 Kb proximal to exon 1 of Kitl and the distal breakpoint within the 3’ untranslated region of Kitl (Fig. 1A; ref. 18). Preliminary sequence analysis of the Slgb deletion map suggested that Kitl may be the only gene deleted by this mutation. Therefore, the Slgb mutation provides a means to test the influence of a Kitl null deletion on TGCT susceptibility independent of alterations in other genes.
In this report, we tested the influence of heterozygosity for the Slgb mutation on spontaneous TGCT incidence in 129/Sv males and fine mapped the Slgb deletion breakpoints. An increase in TGCT incidence was observed in Slgb/+ heterozygotes. Mapping of the Slgb deletion breakpoints revealed no other genes or obvious functional elements within the deletion interval, suggesting that partial deficiency of Kitl increases TGCT susceptibility. We propose that soluble KITL maintains TGCT susceptibility at 129/Sv baseline frequencies in Slgb/+ heterozygous mutant mice and that Kitl is haplosufficient for PGC development in mice with the W mutations.

Materials and Methods

Mice. Mice (129S1/SvImJ; JR002448) were obtained from the Jackson Laboratory. Heterozygous KitlSlgb/Slgb mutant mice were obtained from the research colony of M.A. Bedell (Department of Genetics, University of Georgia, Athens, Georgia; ref 24, 25). Before the tumor survey, the KitlSlgb mutation was backcrossed for at least 10 generations onto 129S1/SvImJ to establish a 129S1/SvImJ-KitlSlgb/Slgb (Slgb/+ congenic strain. Homozygous Slgb/+ embryos were obtained from timed matings of heterozygous mutant mice. Embryonic day 0.5 (E0.5) was assumed to be noon of the day the vaginal plug was observed, and embryos were then collected at E18.5. Homozygous Slgb/Slgb embryos die after E15.5 (18).

Genotyping. A light coat color on the belly and pink tips of the tail and digits on an otherwise normally pigmented mouse distinguished Slgb/Slgb from their wild-type siblings (12, 15). Genomic DNA was isolated from embryo DNA. Sequence information was obtained from the University of California Santa Cruz (UCSC) Genome Browser using National Center for Biotechnology Information (NCBI) Mouse Build 37.1

Slgb tumor survey and statistics. Crosses between 129/Sv and 129/Sv–KitlSlgb/Slgb, in both parental directions, were used for the TGCT surveys. Male offspring were necropsied at ages 4 to 6 wk, and testes were visually examined for tumors. \( \chi^2 \) contingency tests were used to test for statistical differences between the number of affected wild-type and Slgb/+ males, and parent-of-origin effects on TGCT susceptibility.

Slgb deletion mapping. The distal Slgb breakpoint was previously identified to be within the 5′ UTR at bp position 5,287 of the Kitl transcript (18, 26). To identify the proximal breakpoint of the Slgb deletions, we used a genotyping and sequencing strategy (21). Primer sets were designed to PCR amplify sequences 60, 50, 40, and 30 kb proximal to the start of Kitl. A PCR product was generated for all primer sets using wild-type adult DNA, but homozygous Slgb/Slgb mutant embryo DNA failed to generate a product with the 30-kb primer set, placing the proximal breakpoint between −40 and −30 kb of Kitl. New primer sets were designed at 1-kb intervals between −40 and −30 kb of Kitl, and an additional round of PCR further narrowed the region of the proximal breakpoint to between −38.3 and −37.2 kb. PCR was then performed on Slgb/Slgb homozygous and wild-type DNA with primers 5′-CACAGTTAAATATGCACAGCA-3′ (forward; up-stream of the 5′ breakpoint) and 5′-ATCAAAAGGTCCGCGGATA-3′ (reverse; downstream of the 5′ breakpoint) to amplify across the deletion. A 463-bp product was generated from only the Slgb/Slgb homozygous DNA. Sequencing of this PCR product defined the boundaries of the Slgb deletion.

Sequence analysis. Sequence analysis of the Ensembl, 2 NCBI,3 and UCSC mouse genome databases was used to identify transcripts and hypothetical proteins within the deletion intervals of the Steel mutations. The Vista human/mouse/rat Genome Browser4 and the UCSC Genome Browser vertebrate multiz alignment & conservation track were used to identify conserved extra-genic sequences within the Slgb deletion interval (27, 28).

Results

Slgb increases the frequency of affected males in 129/Sv mice. If Slgb (−120 kb deletion) increases the number of affected males as do Sl and Slj (−973 kb and −650 Kb deletions, respectively; Table 1), the candidate region that controls TGCT susceptibility in Steel mutant mice would be significantly reduced. We therefore measured the TGCT incidence in Slgb/+ mutant and wild-type male siblings. TGCT incidence was significantly increased (−2-fold) in Slgb/+ mutant males compared with wild-type controls (Table 2). Therefore, our Slgb/+ tumor survey reduces the critical interval for TGCT susceptibility in Steel mutant mice to −120 Kb.

Additionally, we tested the influence of the parent-of-origin of the Slgb mutation on TGCT incidence in Slgb/+ and wild-type offspring. Parental factors did not affect susceptibility in Slgb/+ offspring as TGCT incidence was similar irrespective of the parental origin of the Slgb mutation (Table 3). By contrast, the parent-of-origin of the Slgb mutation influenced TGCT susceptibility in wild-type offspring, with TGCT incidence being significantly lower when the male parent was heterozygous for Slgb (Table 3). A more detailed analysis of this effect will be reported elsewhere.

Haploinsufficiency of Kitl. We mapped and sequenced the breakpoints of the Slgb deletion to identify candidate TGCT susceptibility genes. The Sl mutation spans several genes, including Kitl, and several predicted functional elements, including two putative Ensembl miRNAs distal to Kitl and several hypothetical

| Table 1. TGCT incidence in Kitl (Sl) and Kit (W) heterozygous mutant mice |
|-----------------|----------------|
| Mutation | Sample size* |
| | | Wild-type (+/+) | Mutant (M+) |
| Sl/+ | 280 | 2.0 | 14.0 |
| Sl/+ | 2,389 | 2.5 | 8.9 |
| Sl/+ | 1,614 | 5.0 | 5.0 |
| W/+ | 650 | 2.0 | 2.0 |
| W/+ | 122 | 2.0 | 0 |
| W/+ | 232 | 2.0 | 0 |
| W/+ | 896 | 2.0 | 3.0 |

**NOTE:** L.C. Stevens and D.S. Varum (Sl/+ data, personal communication; ref. 6).

*The relative numbers of wild-type and mutant mice were not published or available for these crosses. Therefore, statistical significance was not calculated.

1 http://genome.ucsc.edu
2 http://www.ensembl.org
4 http://pipeline.lbl.gov

---

1 http://pipeline.lbl.gov
2 http://www.ensembl.org
4 http://pipeline.lbl.gov

---

Downloaded from cancerres.aacrjournals.org on April 20, 2017. © 2008 American Association for Cancer Research.
Deletion of Kitl Increases TGCT Susceptibility

proteins (Fig. 1A; ref. 21). The SlJ mutation also deletes the two putative Ensembl miRNAs and several hypothetical proteins (Fig. 1A; ref. 18). Additionally, the proximity of the deletion breakpoints to neighboring genes may affect their expression. Based on the rough map of the ~120 Kb Slgb deletion, it seemed that Kitl is the only gene affected by the mutation (Fig. 1A; ref. 18).

To fine-map the breakpoints for the Slgb mutation, we used a combination of PCR and sequencing. The Slgb deletion breakpoints were mapped to bp 99,440,172 and 99,563,881 of mouse chromosome 10 (Fig. 1B). This 123-Kb deletion starts 38 Kb proximal to exon 1 of Kitl and ends within its 3'UTR. Sequence analysis did not reveal any other genes, transcripts, or expressed sequence tags within the Slgb interval in the mouse or other mammalian species. The closest annotated sequences to the proximal end of the Slgb deletion are a hypothetical protein, a ribosomal protein L17-like pseudo-gene (XM_001480394), and citrate synthase-like protein (Csd), −112 Kb and −220 Kb from the Slgb breakpoint, respectively.

The VISTA program and UCSC genome browser revealed several cross-species conserved extragenic elements within the 38 Kb deletion. Sequence analysis did not reveal any other genes, transcripts, or expressed sequence tags within the Slgb interval in the mouse or other mammalian species. The closest annotated sequences to the proximal end of the Slgb deletion are a hypothetical protein, a ribosomal protein L17-like pseudo-gene (XM_001480394), and citrate synthase-like protein (Csd), −112 Kb and −220 Kb from the Slgb breakpoint, respectively.

Figure 1. Mapping of the Slgb deletion.

A, comparison and extent of several mutations of the Steel locus. Schematics of murine chromosome 10 of wild-type, Steel (Sl), Steel J (SlJ), Slgb, and Steel Dickie (Sld) alleles are shown with the centromere (●) on the left and the telomere on the right. The size of each deletion is indicated in kilobases. The exact breakpoints for Slgb are unknown. Arrows, known genes [Csl, kit ligand (Kitl), transmembrane and tetratricopeptide repeat (Tmtc3), and centrosomal protein Cep290 (Cep290)] and two RIKEN clones (4930430F08Rik and 1700017N19Rik); vertical lines, two putative Ensembl microRNAs (ENSMUSG00000077043 and ENSMUSG00000077034); dashed lines, locations of several hypothetical proteins. B, fine mapping of the Slgb deletion breakpoints. A 463-bp PCR product that spans the Slgb deletion was sequenced. Boxed nucleotides indicate the position of the 5' and 3' breakpoints (chromosome 10 bp 99,440,172 and 99,562,881, respectively, of NCBI Mouse Build 37), with 122,708 bp of genomic sequence deleted between these two breakpoints. Alignment of the Slgb deletion sequence with the C57BL/6J reference sequence identified six single nucleotide polymorphisms (SNP).

Discussion

The Kitl isoforms differentially influence TGCT susceptibility in mice. An increase in TGCT incidence was observed in 129/Sv mice harboring the Slgb deletion of the Steel locus. Furthermore, sequencing of the Slgb deletion breakpoints revealed that Kitl is the only conventional gene deleted by this mutation. These results suggest that Kitl is the TGCT modifier at the Steel locus. Interestingly, only the Kitl null deletions (Sl, SlJ, and Slgb) influence TGCT susceptibility on the 129/Sv inbred background. The SlJ mutation, from which soluble Kitl is expressed, does not influence TGCT susceptibility. The contrasting effects of the various Steel mutations on TGCT susceptibility suggest that the transmembrane isoform of Kitl has unusual and specific effects on PGC development.

Both Kitl isoforms are biologically active but may have distinct functional significance in the survival (transmembrane Kitl),...
Parent-of-origin effects of the Slgb mutation on wild-type TGCT incidence. The parent-of-origin effect of the Slgb mutation on wild-type tumor incidence in our Slgb crosses was unexpected. Recently, we showed that genetic modifiers of TGCTs interact across generations to influence TGCT susceptibility (transgenerational epistasis; ref. 13). These interactions represent a novel mode of epigenetic inheritance and may account for the difficulties in identifying TGCT susceptibility genes in mice and humans. Influences of parental genotype on wild-type TGCT incidence have not, however, been previously reported for single genetic modifiers. These parental effects have either not been tested, reported, or do not occur for most TGCT modifiers. We are currently in the process of replicating the Slgb tumor survey to determine whether the parent-of-origin effect observed in the Slgb survey is a common feature of Steel null mutations.

KIT haplosufficiency. Most of the pleiotropic effects observed in Steel homozygous and heterozygous mutant mice are also observed in mice with the various W mutations of the Kit receptor. However, unlike the Steel mutations, none of the tested W mutations increased TGCT susceptibility in heterozygous males. PGC development in heterozygous Slgb and W mutation mice may explain this difference. Heterozygous Steel mutant mice have reduced numbers of PGCs, due to migration and proliferation deficiencies, and a defect in spermatogenesis in the adult testis (15, 21, 33). By contrast, heterozygous W mutant mice have a statistically similar number of PGCs in the developing genital ridge and type A spermatogonia in the adult testis compared with wild-type littermates (34, 35). Therefore dosage of Kit but not Kit affects PGC development. Because TGCTs initiate during embryogenesis, it is therefore not surprising that heterozygosity for W mutations does not modify TGCT susceptibility.

Abnormalities in PGC development and TGCT susceptibility. The mechanism by which the Steel loss-of-function mutations increase TGCT susceptibility is unknown. It is possible that the PGC migration defect of Steel mutant mice contributes to TGCT formation. Transmembrane-bound KITL is expressed by somatic cells along the route of PGC migration and the genital ridge releases chemoattractants, such as soluble KITL, to guide PGCs toward the developing gonad (30, 36–38). When migratory signals are disrupted and male PGCs localize to ectopic tissues, they undergo abnormal developmental changes and initiate apoptosis in response to local stimuli (39, 40). It is possible that in heterozygous null mutant males, disruption of KITL signaling delays germ cell migration into the genital ridge, exposes PGCs to signals outside the developing gonad, and enhance tumorigenic potential after localization into the embryonic testes.

Alternatively, PGCs with altered migration patterns may become apoptotic before entering the genital ridge, resulting in decreased PGC numbers within the developing testis, as is observed in Steel mutant mice (15, 21, 33). A decreased ratio of germ cells to somatic cells within the developing testis may increase the availability of growth factors produced by the somatic cells and alter the developmental fate of the PGCs that localized to the genital ridge. Because Steel mutations act as modifiers of a preexisting susceptibility to TGCTs in 129/Sv inbred mice, it is likely that the effects of delayed migration or altered germ cell to somatic cell ratios are specific to the 129 genetic background.

KIT mutations in human TGCTs. KIT is one of several candidate TGCT susceptibility genes located within a region of human chromosome 4 (4q12) that is frequently amplified in seminomas but not nonseminomas (41, 42). Somatic mutations that constitutively activate the KIT receptor are also associated with seminoma initiation but are extremely rare in nonseminomas and have a statistically similar number of PGCs in the developing genital ridge and type A spermatogonia in the adult testis compared with wild-type littermates (34, 35). Therefore dosage of Kit but not Kit affects PGC development. Because TGCTs initiate during embryogenesis, it is therefore not surprising that heterozygosity for W mutations does not modify TGCT susceptibility.

Abnormalities in PGC development and TGCT susceptibility. The mechanism by which the Steel loss-of-function mutations increase TGCT susceptibility is unknown. It is possible that the PGC migration defect of Steel mutant mice contributes to TGCT formation. Transmembrane-bound KITL is expressed by somatic cells along the route of PGC migration and the genital ridge releases chemoattractants, such as soluble KITL, to guide PGCs toward the developing gonad (30, 36–38). When migratory signals are disrupted and male PGCs localize to ectopic tissues, they undergo abnormal developmental changes and initiate apoptosis in response to local stimuli (39, 40). It is possible that in heterozygous null mutant males, disruption of KITL signaling delays germ cell migration into the genital ridge, exposes PGCs to signals outside the developing gonad, and enhance tumorigenic potential after localization into the embryonic testes.

Alternatively, PGCs with altered migration patterns may become apoptotic before entering the genital ridge, resulting in decreased PGC numbers within the developing testis, as is observed in Steel mutant mice (15, 21, 33). A decreased ratio of germ cells to somatic cells within the developing testis may increase the availability of growth factors produced by the somatic cells and alter the developmental fate of the PGCs that localized to the genital ridge. Because Steel mutations act as modifiers of a preexisting susceptibility to TGCTs in 129/Sv inbred mice, it is likely that the effects of delayed migration or altered germ cell to somatic cell ratios are specific to the 129 genetic background.

KIT mutations in human TGCTs. KIT is one of several candidate TGCT susceptibility genes located within a region of human chromosome 4 (4q12) that is frequently amplified in seminomas but not nonseminomas (41, 42). Somatic mutations that constitutively activate the KIT receptor are also associated with seminoma initiation but are extremely rare in nonseminomas and
pediatric teratomas (42–45). By contrast, deletions of chromosome 12q22, where Kit is located, are associated with nonseminomas but are extremely rare in seminomas (46, 47). In one series of studies, up to 40% of TGCTs showed loss of heterozygosity for a polymorphic marker (D12S7) closely associated with Kit (47, 48). Interestingly, 4q12 amplifications, 12q22 deletions, and activating Kit mutations are rare in TGCT precursor lesions, intratubular germ cell neoplasia (ITGCN); refs. 41, 49. Therefore, modulation of the KIT/KitL signaling cascade is most likely not involved in TGCT initiation but, instead, influences the ultimate developmental fate of ITGCN. Because mouse TGCTs are teratomas and teratocarcinomas, our data and those from humans suggest that a decrease in KIT/KitL signaling, through a gene dosage effect of Kit, supports development of differentiated tumors (teratomas or nonseminomas), whereas activation of Kit/KitL signaling supports development of less differentiated seminomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank L.C. Stevens and D.S. Varmus for sharing their unpublished results and Mary Bedell for providing the Sldg mutant mice.

References


www.aacrjournals.org 5197 Cancer Res 2008; 68: (13). July 1, 2008

Received 2/29/2008; revised 4/14/2008; accepted 4/15/2008.

Grant support: NIH grants CA75056 and CA119623.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. We thank L.C. Stevens and D.S. Varmus for sharing their unpublished results and Mary Bedell for providing the Sldg mutant mice.
Loss of the Transmembrane but not the Soluble Kit Ligand Isoform Increases Testicular Germ Cell Tumor Susceptibility in Mice

Jason D. Heaney, Man-Yee J. Lam, Megan V. Michelson, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/68/13/5193

Cited articles
This article cites 48 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/68/13/5193.full.html#ref-list-1

Citing articles
This article has been cited by 14 HighWire-hosted articles. Access the articles at:
/content/68/13/5193.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.