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Genetic mapping of a *Ptch1*-associated rhabdomyosarcoma susceptibility locus on mouse chromosome 2

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Abstract

Mutations in the *Patched* (*Ptch1*) gene are responsible for various familial and sporadic cancers. $Ptch1^{neo67/+}$ mice, in which exons 6 and 7 are deleted, show genetic background-dependent susceptibility to the development of muscle tumors resembling human rhabdomyosarcoma (RMS); BALB/c (BALB) is a susceptible strain whereas C57BL/6 (B6) shows resistance. A genome-wide linkage analysis was carried out using $Ptch1^{neo67/+}$ mice produced from B6 × (BALB × B6) backcrosses to identify loci involved in the control of RMS susceptibility. Quantitative trait locus mapping with the censored tumor latency time as the quantitative parameter was used to detect a significant RMS susceptibility modifier locus, *Parms1* (*P*atched-*A*ssociated *R*MS 1), on chromosome 2 between D2Mit37 and D2Mit102 (LRS = 10). A Kaplan-Meier survival curve revealed that mice with the B6/BALB genotype develop tumors more frequently and much faster as compared to mice homozygous for the B6 allele ($P = 0.02$). Additional loci not reaching linkage significance were also detected for medulloblastoma resistance.

Keywords: Rhabdomyosarcoma; Ptch1; Tumor-suppressor gene; Linkage analysis

Introduction

Rhabdomyosarcoma (RMS) is a tumor of the striated skeletal muscle and is the most common soft tissue tumor in children. Approximately 65% of cases are diagnosed in children younger than 6 years of age and the mortality is 40% [1]. The early diagnosis of this tumor could be beneficial to improve therapeutic success and prognosis. Some RMSs are associated with distinct inherited cancer syndromes and family histories can help pediatricians identify tumors at an early stage. For example, RMS is associated with Li-Fraumeni and Gorlin syndromes that are caused by germline mutations in the tumor-suppressor genes *TP53* and Patched *(PTCH1)*, respectively [2–4]. For Costello syndrome, associated with an estimated 10% incidence of RMS [5,6], the genetic defect is still unknown. Although these rare familial cancer syndromes have contributed greatly to our understanding of the central processes of rhabdomyosarcomagenesis, the vast majority of RMS cases are not associated with familial syndromes. Rather, nonfamilial RMS cases are probably the result of interactions of polymorphic susceptibility genes with a variety of environmental factors [7, 8].

In man, RMS susceptibility genes will be difficult to identify since they do not segregate as single Mendelian traits. Only association studies using linkage disequilibrium between polymorphisms and cancer susceptibility in large populations could circumvent this problem. However, performing genome-wide association studies in humans requires assaying tens- to hundreds-of-thousands of polymorphic loci in sufficient numbers of affected individuals, a difficult task for rare diseases like RMS. To overcome these

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methodological problems, mouse models are commonly used for studying polygenic or incompletely penetrant traits like mapping and identifying genes that modify cancer risk [9–11].

We used the *Ptch1*$^{neo67/+}$ mouse model of Gorlin syndrome to identify loci that are involved in susceptibility to RMS. Dependent on the genetic background, these mice spontaneously develop RMS and/or medulloblastoma (MB) [12,13]. The B6 strain is genetically susceptible to MBs, whereas it is completely resistant to RMS. In contrast, RMSs develop in up to 9% of mice on the outbred CD1 stock [12] and in more than 50% of F1 hybrids between CD1 and BALB mice by 9 months of age (unpublished data). We investigated the segregation of RMS and MB susceptibility in *Ptch1*$^{neo67/+}$ N2 backcross progeny between the MB-susceptible B6 and the RMS-susceptible BALB strains. Our analysis localized a RMS susceptibility locus near D2Mit42, between D2Mit37 and D2Mit102.

Results

Characterization of tumor phenotypes in heterozygous Ptch1$^{neo67/+}$ *mice*

The parental mice used in this study were on a B6 background and harbored a heterozygous chromosomal region containing the targeted *Ptch1*$^{neo67/+}$ mutation engineered on the 129Sv background [12]. Seventy-two percent of B6-*Ptch1*$^{neo67/+}$ mice develop MB within the first 300 days of life (Table 1; Fig. 1a), whereas none of the animals developed RMS (Table 1; Fig. 1b). In a cohort of 62 BALB × B6 F1-*Ptch1*$^{neo67/+}$ mice, 5 mice (8%) developed MB and 41 mice (66%) developed soft tissue tumors (STTUs), 39 (63%) of which were histologically confirmed as RMS. Two (3%) of the F1-*Ptch1*$^{neo67/+}$ mice developed both types of tumors (Table 1). This result implies that loci exist either when dominant BALB alleles confer MB resistance and RMS susceptibility or when recessive B6 alleles confer MB susceptibility and RMS resistance.

In order to map the locus that confers RMS and MB susceptibility, BALB × B6 F1-*Ptch1*$^{neo67/+}$ males were backcrossed to B6 females to obtain segregating N2 progeny. Eighty-nine heterozygous *Ptch1*$^{neo67/+}$ N2 offspring were monitored for tumor formation over a period of

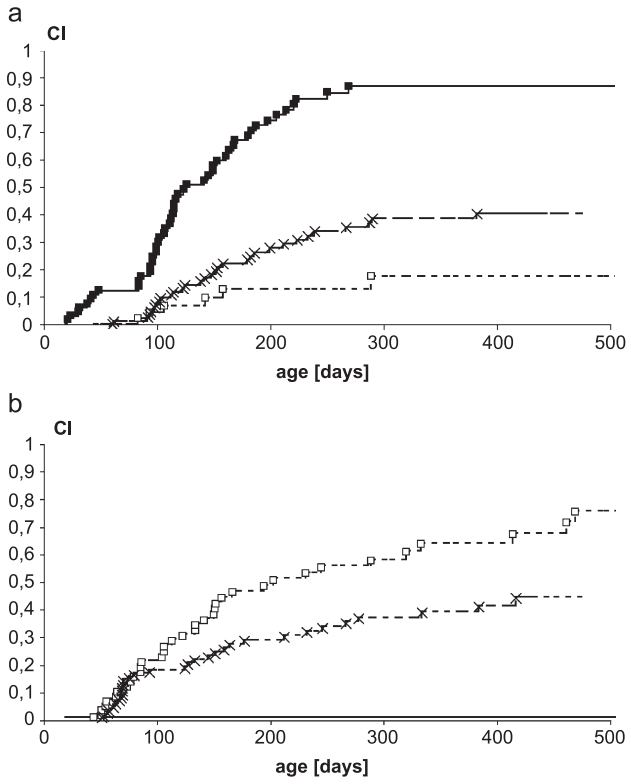

Fig. 1. Tumor incidence in B6-*Ptch1*$^{neo67/+}$(■), BALBxB6 F1-*Ptch1*$^{neo67/+}$(□), and B6×(BALB×B6)-*Ptch1*$^{neo67/+}$N2-backcross (X) mice. (a) MB incidence and (b) RMS incidence of the same cohorts. (CI: cumulative incidence corrected for competing causes of death).

420 days. Animals surviving the study were sacrificed and closely examined for nonvisible tumors. Altogether, 40 animals had developed STTUs and 32 of them turned out to be of RMS histology. The myogenic component of the remaining eight tumors was not clear-cut. Additionally, 28 animals died of medulloblastoma and 6 of these were diagnosed with RMS at autopsy (Table 1).

Linkage analysis of tumor-type modifiers

A whole-genome scan was performed using 97 informative genetic markers with 89 N2 mice. Based on the phenotypic difference between the B6-*Ptch1*$^{neo67/+}$, *Ptch1*$^{neo67/+}$ F1 and the N2 cohorts (Figs. 1a and 1b), any loci contributing at least 20% of the phenotypic variance will be detected with greater than 80% statistical power

Table 1
Tumor phenotype of BALB×B6 F1-*Ptch1*$^{neo67/+}$ and B6 × (BALB × B6)-N2-*Ptch1*$^{neo67/+}$ mice and the parental strains BALB and B6

Phenotype	F1*Ptch1*$^{neo67+}$ (62 mice)		N2*Ptch1*$^{neo67/+}$ (89 mice)		B6*Ptch1*$^{neo67/+}$ (69 mice)		BALB (40 mice)[a]		B6[b]
MB only	5	8%	22	25%	50	72%	0	0%	0%
RMS only	39	63%	26	29%	0	0%	0	0%	0%
MB+RMS	2	3%	6	7%	0	0%	0	0%	0%
Other tumors	5	8%	9	10%	0	0%	2	5%	~10%
Tumor free	11	18%	26	29%	19	28%	38	95%	~90%

[a] The mice were observed over a period of 550 days.
[b] Data are derived from the Jackson Mouse tumor database (http://www.informatics.jax.org/external/festing/mouse/docs/C57BL/6.shtml).

using this population size. This calculation is based upon equations developed from simulation studies [14] and using genome-wide significance levels [15].

Several loci could be identified at which BALB alleles were weakly associated with MB resistance (Table 2). These data suggest that multiple BALB loci contribute to MB susceptibility. The high LOD score for MB resistance associated with B6 homozygosity at D13Mit151 appears paradox but actually results from the fact that the *Ptch1* gene is closely linked to this marker (35 cM according to MGD; 62.4 Mb according to Ensemble). Since the original *Ptch1* knockout allele was generated in a 129Sv ES cell line, we must assume that due to the selection for *Ptch1*$^{neo67/+}$ mice in the present study, all N2 mice were heterozygous 129B6 over the congenic interval surrounding the *Ptch1* locus. However, the markers used could not distinguish a 129Sv from a B6 allele at this locus. Nevertheless we are aware that the possibility exists that loci could be linked to the *Ptch1* locus that were not detected due the selection for the 129Sv-derived targeted *Ptch1* allele.

Linkage analysis was also performed using only N2 mice histologically confirmed to have RMS. This analysis revealed linkage to a single locus on chromosome 2. The highest single-point LOD (1.73) for RMS susceptibility was associated with a BALB/B6 heterozygous genotype at D2Mit42. Of the 32 mice that developed histologically confirmed RMS, 18 were heterozygous B6/BALB and 14 homozygous B6/B6, whereas of 32 long-surviving tumor-free mice 7 were B6/BALB but 25 B6/B6. The LOD score increased to 2.44 if only tumors with a higher differentiation status were used in the analysis (Table 3).

Interval mapping using 11 additional polymorphic markers defined the region between D2Mit37 and D2Mit102 as most likely harboring a locus, *Parms1*, where the B6 allele confers resistance to STTUs (data not shown) or histologically confirmed RMS (Fig. 2). Using a permutation test with the set of genotyped animals from this experiment the threshold for significant linkage was defined for LRS >6.92. As shown in Fig. 2 LRS for linkage between D2Mit37 and D2Mit102 exceeds this value (LRS= 10) and thus linkage is significant. Based on the available data, the size of the mapped interval is 7.5 cM (mouse genome database: http://www.informatics.jax.org/) or 40.04 Mb (Ensemble database: http://www.ensembl.org/Mus_musculus; version v13.30.1 based on the NCBI 30 build). Therefore we cannot exclude the possibility that more than one gene is responsible for this locus. When lifetime tumor-free status was monitored, we observed that only 33% of B6 homozygous mice at this locus develop RMS (median LT = 161.5 days) as compared to B6/BALB heterozygous mice, of which 64% develop RMS (median LT = 86 days). Differences in tumor incidence between the Kaplan-Meier curves are significant (p = 0.02, Gehan's Wilcoxon test). For development of all STTUs the results were similar (data not shown). Thus, 57% of mice homozygous B6 on Chr 2 develop STTUs (median LT = 198 days) whereas 78% of mice heterozygous B6/BALB develop STTUs (median LT = 104.5 days).

Table 2
Putative loci influencing MB development in B6 × (BALB × B6)-*Ptch1*$^{neo67/+}$ mice

Marker	Pos. [cM]	MB		No MB		χ^2	LOD	P
		C/C	B/C	C/C	B/C			
D4Mit235	1.9	19	9	25	36	5.54	1.21	0.019
D6Mit138	0.68	20	8	25	36	7.12	1.55	0.008
D7Mit259	72	18	10	24	37	4.79	1.04	0.03
D9Mit35	52	10	18	38	23	5.46	1.19	0.02
D13Mit151	71	13	15	49	12	10.44	2.27	0.0012

Marker position, mouse number/genotype distribution in each phenotype category, LOD scores, P values, χ^2 values for single-point linkage test.

Table 3
Putative loci influencing RMS development in B6x(BALB×B6)-*Ptch1*$^{neo67/+}$ mice

Marker	Pos. [cM]	RMS		No RMS (>300 days)		χ^2	LOD	P
		C/C	B/C	C/C	B/C			
D2Mit42	47.5	14	18	25	7	7.94	1.73 (2.44)[a]	0.0048
D2Mit398	57.9	13	19	23	9	6.35	1.38	0.01

Marker position, mouse number/genotype distribution in each phenotype category, LOD scores, P values, χ^2 values for single-point linkage test.
[a] For highly differentiated RMS only.

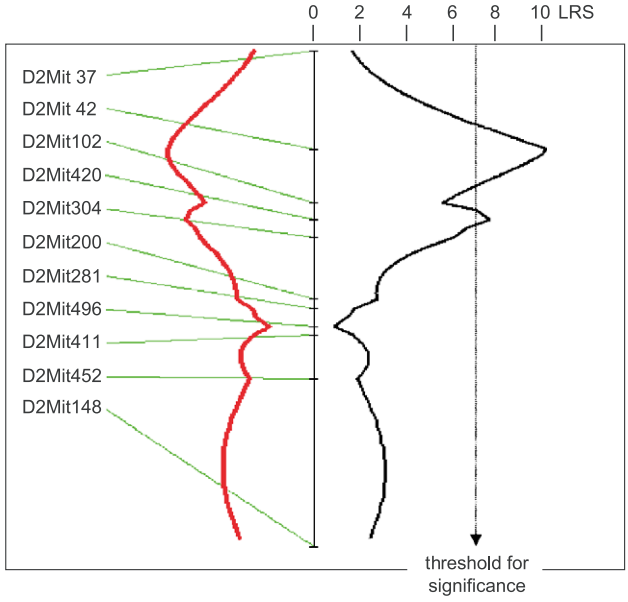

Fig. 2. Location of the RMS susceptibility locus on chromosome 2. QTL interval mapping was performed using the censored normalized latency time of RMS with MapManager QTXb19. The black line gives the likelihood ratio statistics LRS along the interval on chromosome 2 starting from D2Mit37 at 45 cM down to D2Mit148 at 105 cM. The dotted line shows the threshold for significance of linkage as derived by a permutation test. Gray line to the left of the chromosomal axis shows the relative phenotypic effect of a putative susceptibility allele.

Discussion

The studies reported here provide support for the existence of modifier loci that can influence tumor susceptibility in *Ptch1*$^{neo67/+}$ mice. The first line of evidence came from the distinct phenotypes of B6-*Ptch1*$^{neo67/+}$ and the CD1-*Ptch1*$^{neo67/+}$ mice [12] and of F1-*Ptch1*$^{neo67/+}$ hybrids between CD1 and BALB strains. Whereas B6-*Ptch1*$^{neo67/+}$ mice develop MBs and are completely resistant to rhabdomyosarcomagenesis, the CD1 and BALB strains develop both types of tumors. These data show that besides the major tumor-susceptibility locus *Ptch1*, which affects tumor development due to its key role in cell growth and differentiation, other genetic loci exist that modulate the spectrum of *Ptch1*$^{neo67/+}$-associated tumors.

In order to identify these loci B6 × (BALB × B6) N2 mice were used, all of which were heterozygous for the *Ptch1*$^{neo67/+}$ allele and thus predisposed to develop either RMS or MB tumors. For the initial genome-wide scan, a set of 97 microsatellite markers was employed. Since the average genome coverage was 14 cM, the set was adequate for an initial genome-wide screen [16].

Our data show that multiple loci seem to influence MB susceptibility. However, because the LOD scores and corresponding *P* values are only weakly suggestive for each single locus, a detailed analysis would not be appropriate at this stage without a confirmatory backcross or other supporting mapping techniques like analysis in recombinant inbred lines [17]. In contrast to the multiplicity of loci influencing MB susceptibility, only a single locus on chromosome 2, *Parms1*, between D2Mit37 and D2Mit102, was identified harboring a RMS susceptibility modifier locus (Fig. 2). Whereas the F1 analysis could not exclude the possibility that the same locus confers both MB resistance and RMS susceptibility in BALB, thus having a pleiotrophic effect on both tumor types, linkage analysis using a segregating N2 population shows that there are indeed distinct loci for MB resistance and RMS susceptibility.

The LOD score of 2.44 for D2Mit42 was used as a guide for more in-depth analysis of the *Parms1* locus and an additional 11 markers for interval mapping were employed. In order to determine significance threshold for the data, a permutation analysis was performed [18,19], which is the currently accepted method [20]. The resulting LRS as well as the association between tumor development and the B6/BALB genotype showed that the identified QTL was significant.

The effect of the BALB RMS susceptibility allele at the chromosome 2 locus is to shorten RMS latency time (Fig. 3). Since we did not notice any systematic differences in morphology or differentiation between tumors from B6/B6 homozygous and B6/BALB heterozygous mice, the *Parms1* locus appears to be involved in the early stages of tumor induction, rather than in later stages of tumor progression.

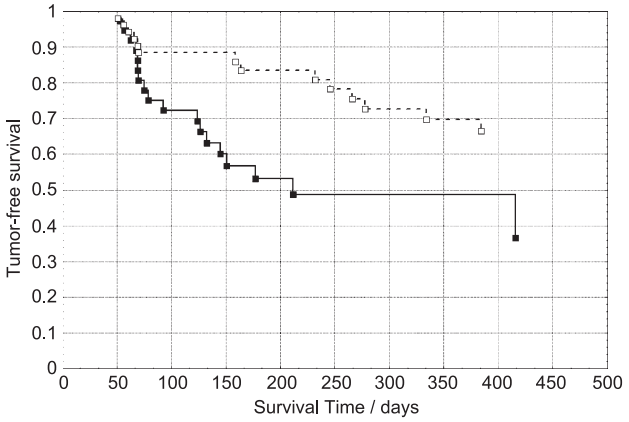

Fig. 3. Lifetime RMS monitoring of B6×(BALB×B6)-*Ptch1*$^{neo67/+}$ mice. Thirty-three percent of mice homozygous B6 at D2Mit42 (□) develop histologically confirmed RMS (median LT = 161.5 days) whereas 64% of B6/BALB heterozygous mice (■) develop RMS (median LT = 86 days). Tumor-free survival corrected for confounding causes of death was calculated using Kaplan-Meier function in STATISTICA6 software.

The RMS susceptibility region between the two flanking markers spans approximately 7.5 cM on mouse chromosome 2. According to a genome built from the Mouse Genome Sequencing Consortium (http://www.ensembl.org/Mus_musculus/), this region contains approximately 350 known or predicted genes. However, around 180 of these genes are part of a cluster of olfactory receptors and most likely not responsible for tumor susceptibility. Nonetheless, there are several strong candidates for the RMS modifier. These include genes that are involved in DNA repair such as *Pms1* and *Rad51*, that are known to be involved in cancer development [21,22]. Another strong candidate is the tumor-suppressor gene *Wt1*. In humans, mutations in Wt1 lead to Wilms' tumor in addition to being involved in a variety of other solid tumors [23]. Interestingly, *Wt1* regulates *Igf2*, a gene that is highly overexpressed in *Ptch1*-associated tumors [24,25]. This region of mouse chromosome 2 also harbors several other cancer modifier loci influencing lung and colon cancer susceptibility [26–28]. Whether any of these are the same or different than *Parms1* will require additional cosegregation analysis.

The mouse RMS candidate locus between markers D2Mit37 and D2Mit102 shows conserved synteny to human chromosomes 2q31–q33, 11p11–p13, and 15q14–q15. All regions are associated with development of human RMS and have been reported to show strong chromosomal gains or losses in human RMS by CGH analysis [29,30]. Taken together with our data, this provides strong evidence for one or more RMS modifiers on mouse chromosome 2.

In the future, knowledge about genetic polymorphisms that influence individual risk of children to develop RMS (or MB) will aid early identification of these tumors and thus improve the prognosis for these highly malignant childhood tumors.

Material and methods

Animals

The 129Sv- *Ptch1*$^{neo67/+}$ mutation [12] was bred for at least 10 generations onto the C57BL/6NCrlBR (B6) background to obtain B6-*Ptch1*$^{neo67/+}$ mice (available as *Ptch*$^{tm1Zim/+}$ from EMMA). The genotypes of the mice were determined by PCR as described [12]. The mapping experiments were performed using inbred BALB/cByJ (BALB) mice purchased from Charles River Laboratories, L'Arbresle, France, and C57BL/6NCrlBR (B6) mice purchased from Charles River Laboratories, Sulzfeld, Germany. To obtain the F1 generation, B6-*Ptch1*$^{neo67/+}$ males were mated to BALB females. The N2 backcross progeny were then produced by mating *Ptch1*$^{neo67/+}$ F1 males to wild-type B6 females. All mice were treated and housed in accordance with the German animal protection laws and monitored regularly by the campus veterinarian.

Scoring of tumors

Mice were monitored for tumor formation weekly. Animals were sacrificed by CO_2 asphyxiation when moribund, for example, because of development of medulloblastoma, or when visible muscle tumors reached a size of 1 cm. All animals were closely examined after death for additional, nonvisible tumors. The identity of the tumors as RMS or MB was confirmed by a trained pathologist using hematoxylin and eosin (H & E)-stained sections.

Genetic analysis

A total of 89 N2 animals were used in this study. DNA was prepared from tails by overnight incubation at 55°C in STE buffer (50 mM Tris, pH 8; 100 mM NaCl; 1% SDS; 1 mM EDTA; 500 μg/ml proteinase K) followed by phenol-chloroform and chloroform-isoamyl-alcohol extraction, precipitation with ice-cold 100% ethanol, and washing with 70% ethanol. For the initial genome-wide scan 97 microsatellite markers from the MIT and CIDR databases were genotyped by PCR. The average genome coverage of the markers used was 14 cM. Eleven additional markers were used for interval mapping between the markers D2Mit37 and D2Mit452 that gave the strongest linkage in the initial genome scan. The amplified PCR fragments were visualized by EtBr staining following electrophoresis through 4% agarose gels. Marker positions are based on the Mouse Genome Database. For each 96-well plate, duplicates of three control DNA samples from B6, BALB, and F1 mice and a negative control were typed. The markers and PCR conditions used for analysis are available upon request.

Modifier mapping and statistics

A whole genome single-point linkage analysis was performed for each marker by comparing the number of mice with B6/B6 and B6/BALB genotypes in the group of mice that developed RMS or MB as compared to those that remained tumor-free and lived longer than 280 days. These numbers were used to calculate LOD scores and P values using a χ^2 test. Thresholds for significant deviation from randomness were corrected for multiple testing applying the Bonferroni formula.

For fine-mapping modifiers of the RMS or MB incidence, each of the 89 N2 mouse was attributed a censored normalized latency time (LTc), which takes into account the limited phenotype information of mice that remain tumor-free but died with other diagnosis. This quantitative parameter was calculated as follows:

$$LT_C = (t_{\max} - t)/t_{\max}$$

(mice dying with the analyzed tumor type)

$$LT_C = -t/t_{\max} \text{ (mice dying of other reasons)},$$

with t being the age at death and $t_{\max}$ being the maximal observed lifetime. LT_C thus runs from –1 to 0 for tumor-free mice and from 0 to+1 for mice developing the specified tumor type, with –1 reflecting the longest tumor-free survival and +1 early arising tumors. For this normalized trait parameter we performed QTL interval mapping using MapManager QTXb19 software [31]. The likelihood ratio statistics (LRS) as derived by this program were evaluated for significance using a permutation test that is part of MapManagerQTX. Permutation tests are more accurate in establishing the appropriate significance thresholds than arbitrary levels [18,19].

To evaluate differences in tumor incidence between mouse strains or genotype groups, Kaplan-Meier curves for tumor-free survival were calculated taking into account confounding causes of death. Differences in tumor incidence curves were tested for significance using Gehan's Wilcoxon test (Statistika 6, StatSoft, Tulsa, OK).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2004.07.002.

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