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Mapping of Melanoma Modifier Loci in *RET* Transgenic Mice

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Transgenic mice carrying the *RET* oncogene under the control of the metallothionein promoter exhibit severe pigmentation of the whole skin and melanocytic tumors. The genetic background influences melanoma development in *RET* mice; founder mice crossed with BALB/c mice show decreased incidence and increased latency of melanocytic tumors, whereas progeny of C57BL/6 mice show the opposite effect. Using partially congenic *RET* mice on a C57BL/6 genetic background (N3/*RET* mice), we studied genetic linkage in (N3/*RET*×BALB/c)×N3/*RET* backcross mice. We mapped three melanoma modifier loci, on chromosome 1 (*Melm1* and *Melm2*) and chromosome 11 (*Melm3*), that are linked with early melanoma incidence and latency. Mapping of *Melm* loci and of five additional regions on chromosomes 6, 8, 9, 12, and 13 indicated allelic imbalance in N3/*RET* mice, with a significant excess of BALB/c alleles, suggesting the presence of additional putative melanoma modifier loci on these chromosomes.

Key words: Melanoma — Genetic linkage — RET — Transgenic mice

Mouse inbred strains show a great variability in spontaneous and chemically induced tumors.[1)] Such mice have provided a tool to map cancer susceptibility/resistance loci and to delineate the complex genetics of inherited predisposition to tumorigenesis in several organs.[2–4)] However, inbred mice do not spontaneously develop melanomas, nor do chemical carcinogens or irradiation induce this tumor type in mice.[5)]

RET transgenic mice represent the first mouse model of melanoma development.[6)] In these mice, skin pigmentation and melanoma phenotypes are caused by *RET* oncogene expression in target cells and not by insertion mutagenesis of the transgene into a target chromosomal region. Indeed, four independent founder mice exhibited the same phenotype, and the *RET* transgene was expressed at high levels in non-tumor melanin-produced cells and in melanomas.[6)] The *RET* transgene encodes an activated tyrosine kinase protein. Overexpression of tyrosine kinase proteins has been implicated in human melanoma[7,8)] and overexpression of the c-Met receptor tyrosine kinase induced melanoma in a transgenic mouse model.[9)]

MATERIALS AND METHODS

Mice and phenotypes Line 304 *RET* transgenic mice, which exhibit severe melanocytic abnormalities and melanomas, originated into the (BALB/c×C57BL/6)×BALB/c genetic background and were maintained in the (C57BL/6×BALB/c)F1 background.[6)] These *RET* mice were crossed three times with C57BL/6 mice to produce a partially congenic line on a C57BL/6 background. After the third cross (N3), mice showed low fertility and early melanoma development, and were therefore maintained by random mating in a closed colony for 10 generations before starting the backcross study. In the colony, selection occurred against mice showing poor fertility or poor health, or developing tumors at early age. Analysis of N3/*RET* mice for the presence of the *RET* transgene failed to detect homozygous animals, indicating that *RET* transgene homozygosity is lethal in this model. All mice used in the present study were therefore heterozygous for the *RET* transgene.

To carry out genetic linkage studies, N3/*RET* mice were crossed to BALB/c mice and the resulting F1 mice (selected for the presence of the *RET* transgene) were backcrossed with N3/*RET* mice; 195 backcross animals carrying the *RET* transgene were used for the genetic linkage analysis. In addition, 28 N3/*RET* mice, including 5 mice that have been used to generate the backcross population, were typed with genetic markers. The incidence and latency of tumors at 200 days of age were scored as melanoma phenotypes. Melanoma incidence and latency were also analyzed in 41 N3/*RET* and in 58 (BALB/c×N3/*RET*)F1 mice, all carrying the *RET* transgene.

Genotype analysis DNA was extracted from the tails of mice using the DNeasy Kit (QUIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. A preliminary genome scanning was carried out in two DNA pools, prepared from 17–20 mice each, chosen from the group that did not develop tumors and from the group with the earliest tumor onset (70–140 days). Two hundred

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sixty-nine microsatellite markers, separated by 10–15 cM and dispersed over the whole mouse genome, were amplified using PCR primers from Research Genetics (Huntsville, AL). Non-radioactive or ^{32}P-labeled PCR was performed and products were analyzed by electrophoresis on 6 to 10% non-denaturing polyacrylamide gels. When differences in allele size were sufficient, PCR products were loaded on agarose gels and visualized with ethidium bromide. When allelic imbalance in a genomic region was detected during analysis of the DNA pools, the same region was re-analyzed in each of the 195 backcross mice, using additional markers. N3/*RET* mice were genotyped with a total of 82 microsatellite markers.

Statistical analysis In backcross mice, the association of marker alleles with tumor incidence was evaluated by Fisher's exact test.[10] Curves for association of marker alleles with melanoma latency were generated by the Kaplan-Meier method and analyzed by log-rank test[10, 11]; the resulting *P* values were transformed to negative logarithms ($-\log P$) for a clearer presentation of the statistical evidence.[12] The linkage between melanoma latency and genetic markers was confirmed by multipoint interval mapping analysis, using MAPMAKER/QTL.[13] In N3/*RET* mice, the predictable proportion of BALB/c alleles on chromosomes not bearing *RET* transgene was 6.25%; thus, at any marker locus a frequency higher than 23% of BALB/c allele was considered as a significant allelic imbalance ($P<0.05$, Fisher's exact test).

RESULTS

Melanoma phenotype Mice of the N3/*RET* line developed melanoma at a very early age, starting from 6 weeks; 85% of the mice had tumors by 28 weeks (200 days) (Fig. 1). Melanomas in backcross mice were most often observed as single tumors of the skin or the eyes. N3/*RET* mice crossed with BALB/c mice gave rise to F1 progeny that showed a melanoma incidence of <2% at 28 weeks of age (Fig. 1). Melanoma incidence was 25% in the (BALB/c×N3/*RET*)×N3/*RET* backcross mice, which showed a tumor phenotype intermediate between those of N3/*RET* and F1 mice (Fig. 1).

Melanoma modifier loci A whole-genome scanning was carried out in two DNA pools prepared from backcross mice that developed no melanomas or early onset melanomas. Three chromosomal regions were associated with the tumor phenotype(s); two of these regions mapped on chromosome 1 and the third mapped on chromosome 11.

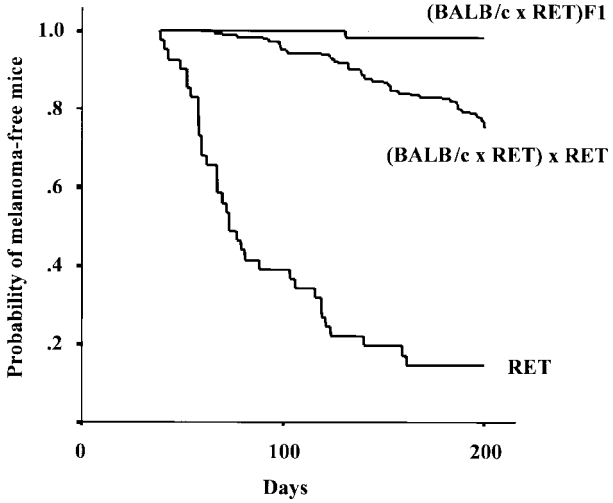

Fig. 1. Kaplan-Meier estimates of melanoma latency in N3/*RET* (n=41), (BALB/c×N3/*RET*)F1 (n=58), and (N3/*RET*×BALB/c)×N3/*RET* (n=195) mice.

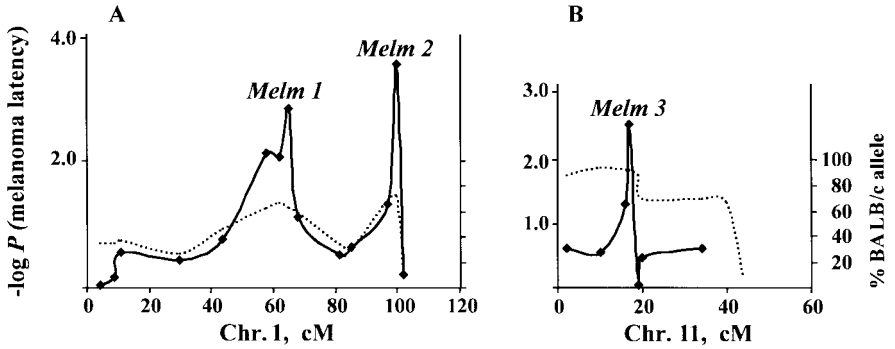

Fig. 2. Genetic linkage mapping (continuous line) and allelic imbalance (dotted line) at *Melm1* and *Melm2* (A) and *Melm3* (B) loci. Distances in centiMorgans (cM) are based on the MGD map (http://www.informatics.jax.org/). Negative logarithms of the statistical *P* values ($-\log P$) were obtained by analysis of Kaplan-Meier melanoma latency curves in backcross mice. Cutoff value for statistical significance ($P<0.05$) of allelic imbalance in N3/*RET* mice (excess of BALB/c alleles) was 23% (see "Materials and Methods"). ◆, a typed genetic marker in the backcross and in the N3/*RET* mice.

Analysis of the whole population of 195 backcross mice confirmed linkage with a melanoma phenotype(s) for all three loci. One peak at *D1Mit494* (65 cM) was associated with early melanoma onset (−log *P*=2.82, Figs. 2, 3) and incidence (*P*=0.004) since 8/61 (13%), 25/95 (26%), and 11/23 (48%) mice of the "11" (BALB/c homozygous), "12" (heterozygous), and "22" (C57BL/6 homozygous) genotypes, respectively, developed melanoma by 200 days (Fig. 3). Interval mapping analysis of melanoma latency assigned a peak lod score=2.5 at the *D1Mit494* marker. We named the locus linked to the *D1Mit494* marker "*Mel*anoma *m*odifier *1*" (*Melm1*). At this locus, the BALB/c strain carried the "resistant" allele, which showed a co-dominant effect, since heterozygous mice showed melanoma incidence and latency intermediate between those of homozygous mice carrying either the BALB/c or the C57BL/6 genotype (Fig. 3). BALB/c homozygous animals were unexpected because of the backcross design of the study, and indicated the presence of residual BALB/c genetic material in N3/*RET* mice.

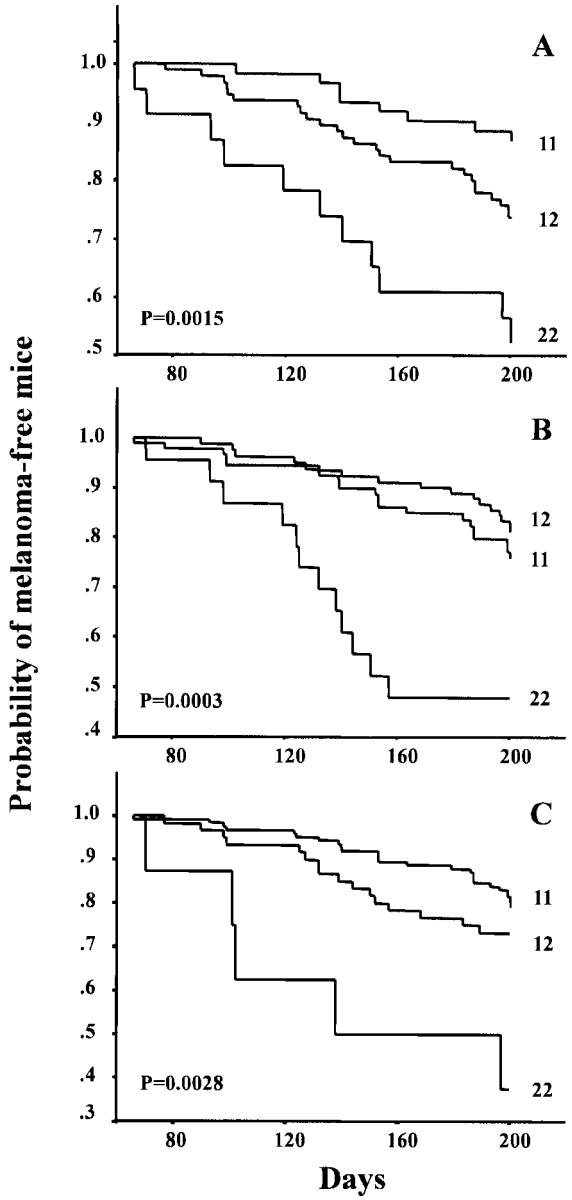

Fig. 3. Genetic linkage of melanoma latency with *D1Mit494* (*Melm1*, A), *D1Mit150* (*Melm2*, B), and *D11Mit231* (*Melm3*, C) markers in backcross mice (allele 1=BALB/c, allele 2=C57BL/6).

The other locus on the same chromosome 1, *D1Mit150*, was distally located at 100 cM, and was also associated with early melanoma onset (−log *P*=3.52, Figs. 2, 3) and incidence (*P*=0.006), since 19/79 (24%), 17/90 (19%), and 12/23 (52%) of mice of genotypes 11, 12, and 22, respectively, developed melanoma by 200 days (Fig. 3). Interval mapping of latency showed a peak lod score=5.4 at 0.8 cM proximal to *D1Mit150*, and a lod=4.2 at *D1Mit150*. We named this locus *Melm2*, and the BALB/c strain carried the melanoma resistance allele, which showed a dominant melanoma resistance effect (Fig. 3).

Tumor latency was also linked to a region of chromosome 11, with a peak around the *D11Mit231* locus (17 cM, −log *P*=2.55 and lod=3.7 by interval mapping). We named this third locus *Melm3*, with the melanoma resistance allele deriving from the BALB/c strain and showing a dominant pattern of inheritance (Fig. 3). However, *Melm3* showed only a borderline statistical association with early melanoma incidence (*P*=0.034), probably because of the small number of mice homozygous for the C57BL/6 allele (*n*=8).

Whole-genome scanning revealed no other chromosomal regions associated with melanoma phenotypes. Nevertheless, 39 additional genetic markers, located on chromosomes 2–7, 9, 10, 12, 17, and 18, were typed in the whole backcross population, since these chromosomal regions are homologous to the human regions where familial studies or loss of heterozygosity in melanomas have indicated the presence of putative melanoma predisposition/suppressor loci.[14–16] No other significant association between genetic markers and melanoma development was found.

Over-representation of BALB/c alleles N3/*RET* mice are expected to carry a residual 6.25% of BALB/c genome, considering the generation of these mice from the line 304 backcrossed three times into the C57BL/6 genetic background and then maintained by random mating. However, whole-genome scanning in backcross mice revealed an unexpected over-representation of the BALB/c alleles at different regions in both DNA pools (from melanoma-resistant and -susceptible mice). These regions mapped on chromosomes 1, 6, 7, 8, 9, 11, 12, and 13, and were re-analyzed in N3/*RET* mice using additional microsatellites

(results shown in Fig. 4 for chromosomes 6, 7, and 13). On chromosome 6, genetic markers spanning from 5 to 74 cM showed that the BALB/c allele in N3/*RET* mice accounted for 41 to 100% of the chromosome, with a 100% peak in the 57–65 cM region. On chromosome 7, genetic markers located near the centromere (from 1 to 11 cM) indicated a large excess (75–90%) of BALB/c alleles, whereas distal markers (from 26 to 54 cM) showed the expected frequency of BALB/c alleles (8–10%). On chromosome 8, genetic markers spanning from 1 to 72 cM showed that the BALB/c allele in N3/*RET* mice ranged from 77 to 100%, with a 100% peak over most of the chromosome, i.e., from 13 to 54 cM. On chromosome 9, genetic markers spanning from 17 to 71 cM showed that the BALB/c allele ranged from 50 to 91%, with a peak region (>70%) on 17–48 cM. On chromosome 12, genetic markers spanning from 19 to 50 cM showed that the BALB/c allele ranged from 39 to 52%. On chromosome 13, genetic markers spanning from 5 to 75 cM showed that the BALB/c allele ranged from 10 to 81%, with a sharp peak region (81%) around 10–16 cM, whereas distal markers retained the expected frequency of BALB/c allele. Analysis of the backcross population confirmed the over-representation of the BALB/c alleles in N3/*RET* mice; almost all backcross animals indeed carried at homozygosity BALB/c-derived alleles in chromosomal regions where peaks of allelic imbalance have been observed in N3/*RET* mice.

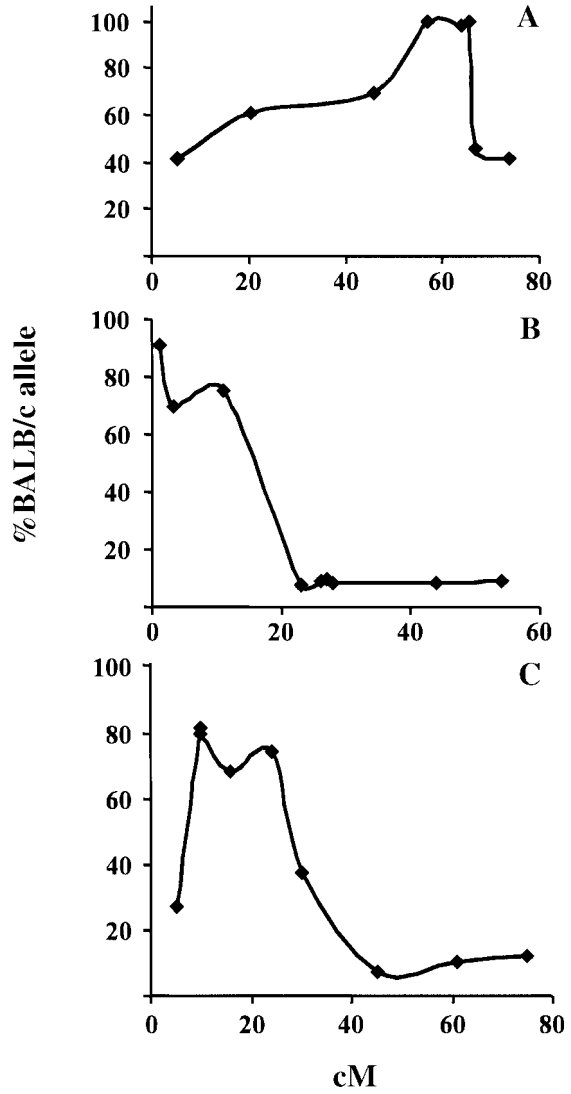

Fig. 4. Allelic imbalance in chromosomes 6 (A), 7 (B), and 13 (C) of N3/*RET* mice. ◆, a typed genetic marker (see also Fig. 2).

DISCUSSION

Our study shows that melanoma development in *RET* transgenic mice can be inhibited by BALB/c-derived alleles at melanoma modifier loci. Three of these loci (*Melm1–3*) map on chromosomes 1 and 11. The mice of the N3/*RET* colony showed an unexpected large excess of the BALB/c genome in the same chromosomal regions, making it unlikely that this allelic imbalance occurred by chance during the maintenance of the colony. In fact, the allelic imbalance was chromosome-specific and region-specific within a single chromosome. Distal regions of chromosomes 7 and 13 contained the expected proportion of the BALB/c allele, whereas the centromeric regions derived mostly from the BALB/c strain (Fig. 4). The melanoma modifier loci (*Melm*) also map to the regions of allelic imbalance, and it may be that mice with BALB/c alleles at these regions have a selection advantage in the form of delayed or inhibited melanoma onset or increased survival. C57BL/6 alleles at these loci may instead cooperate with or over-stimulate *RET* transgene activity and lead to poor fetus survival and/or early melanoma development, representing a negative selection pressure in the N3/*RET* mouse colony. This hypothesis is consistent with the opposite effects observed by crossing *RET* mice with either BALB/c (decreasing melanoma incidence and prolonging latency) or C57BL/6 mice (increasing melanoma incidence and shortening latency).[6, 17]

The *Melm1–3* loci were located on regions of allelic imbalance (Fig. 2), consistent with the derivation of the melanoma resistance alleles from the BALB/c strain. As a consequence, the number of mice homozygous for the C57BL/6 allele at these loci was much lower than that expected in a standard genetic linkage experiment (i.e., 13%, 12%, and 5% at the *D1Mit494*, *D1Mit150*, and *D11Mit231* loci, respectively, vs. 50% expected). Thus, the statistical significance levels we determined for linkage of early melanoma onset/incidence with *Melm* loci most

probably represent an underestimate, due to the reduced number of animals carrying the susceptibility allele (C57BL/6) and expressing the early melanoma phenotype.

The heterogeneity of the N3/*RET* mouse genome due to over-representation of BALB/c alleles enabled us to distinguish the *Melm1* and *Melm2* loci, which are separated on the chromosome 1 by only 35 cM. Standard crosses provide a much lower resolution in this respect, even with a specifically designed method of analysis.[18] Moreover, heterogeneity of these mice led to very narrow linkage peaks for the *Melm* loci, i.e., ~5 cM instead of the typical 15–30 cM. We recently observed the same analogous advantage of genetic heterogeneity of the parental strains in the fine mapping of cancer modifier loci, in a non-inbred mouse model of genetic predisposition to squamous cell skin cancer.[19, 20] The narrow linkage peaks and the consequent short mapping regions of *Melm* loci, provide a first step in gene cloning, either by analysis of candidate genes or by positional cloning, without the need of shortening the linkage regions by marker-assisted congenics.[21]

In humans, the 9p21 region, containing the *p16* gene, is linked to familial melanoma.[15] Our own extensive analysis of mouse chromosome 4, which contains the homologous region, revealed no significant linkage of either the *p16* (*Cdkn2a*) locus or the surrounding loci with melanoma phenotypes (data not shown), even considering that BALB/c mice carry a p16 allele different from that of other strains.[22] These results are consistent with the observation that mice null/null for p16 and p19 fail to develop melanoma.[23]

Candidate genes mapping in the *Melm1* region (http://www.informatics.jax.org/) include *Cmkar4* (67.4 cM, chemokine (C-X-C) receptor 4) and *Inhbb* (64.1 cM, inhibin β-B); the last one is reported differentially expressed in association with metastatic potential of melanoma cells.[24] Candidates mapping close to the *Melm2* region include *Ifi201–204* (95.2 cM, interferon activated genes 201–204), *H25* (100 cM, histocompatibility 25), *Itpkb* (100 cM, inositol 1,4,5-trisphosphate 3-kinase B), *Tgfb2* (101.5 cM, transforming growth factor, β-2), and *Traf5* (105 cM, tumor necrosis factor (TNF) receptor-associated factor 5). In the region of *Melm3*, on chromosome 11, *Egfr-rs* (16 cM, epidermal growth factor receptor-related sequence) and *Il12b* (19 cM, interleukin-12b) are found. Although these genes have biochemical and biological functions consistent with an association with melanoma, proof that any of them are *Melm* genes awaits analysis of strain-specific polymorphism or expression, and functional assays.[2]

The *Melm* loci that we have mapped may modulate melanoma onset and development by interfering with melanoma differentiation, antigen presentation, apoptosis, cell cycle, or by affecting *RET* activity, which in our model induces melanoma. For example, candidate *Melm* loci may encode allelic variants of proteins involved in the *RET* oncogene signal transduction pathway, or of immunological modulants of the *RET* protein.[25] Since *RET* oncogene activation is involved in several diseases,[26–28] cloning of *Melm* candidate genes might represent a step in developing new strategies for melanoma control, as well as for the human pathologies associated with *RET* oncogene activation.

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