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Advances in Brief

Epistatic Interactions between Skin Tumor Modifier Loci in Interspecific (*spretus/musculus*) Backcross Mice[1]

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Abstract

The development of cancer is influenced both by exposure to environmental carcinogens and by the host genetic background. Epistatic interactions between genes are important in determining phenotype in plant and animal systems and are likely to be major contributors to cancer susceptibility in humans. Several tumor modifier loci have been identified from studies of mouse models of human cancer, and genetic interactions between modifier loci have been detected by genome scanning using recombinant congenic strains of mice (R. Fijneman *et al.*, Nat. Genet., *14:* 465–467, 1996; T. van Wezel *et al.*, Nat. Genet., *14:* 468–470, 1996; W. N. Frankel *et al.*, Nat. Genet., 14, 371–373, 1996). We demonstrate here that strong genetic interactions between skin tumor modifier loci can be detected by hierarchical whole genome scanning of a complete interspecific backcross [outbred *Mus spretus X Mus musculus* (*NIH/Ola*)]. A locus on chromosome 7 (*Skts1)* showed a highly significant interaction with *Skts5* on chromosome 12 ($P < 10^{-16}$), whereas additional significant interactions were detected between loci on chromosomes 4 and 5, and 16 and 15. Some of these quantitative trait loci and their interactions, in particular the *Skts1-Skts5* interaction, were confirmed in two completely independent backcrosses using inbred *spretus* strains (*SEG/Pas* and *SPRET/Ei)* and *NIH/Ola.* These results, therefore, illustrate the general use of interspecific crosses between *Mus musculus* and *Mus spretus* for the detection of strong genetic interactions between tumor modifier genes.

Introduction

In a previous study, F1 animals between tumor-resistant *Mus spretus* and tumor-susceptible *Mus musculus* strains were almost completely resistant to skin tumor development after carcinogen exposure. Three significant linkages at loci on chromosomes 5 and 7 were identified by QTL[3] analysis in a large F1 backcross study (1). *Skts3* on chromosome 5 was linked to both promotion and progression, whereas *Skts1* and *Skts2* (chromosome 7) were mainly associated with benign papilloma development with no obvious linkage to development of carcinomas. Further analysis using negative binomial regression identified an additional seven novel loci associated with benign tumor multiplicity or carcinoma incidence. Interestingly, a subset of these loci was associated with time of survival of carcinoma-bearing mice (2).

Demant and coworkers (3, 4) have exploited RCSs to detect several genetic interactions involved in susceptibility to carcinogenesis in the mouse colon and lung. A series of RCSs were developed, each of which has 87.5% of the genome from a donor strain and 12.5% from a recipient. The resultant reduction in genetic complexity (5) has allowed the identification of epistatic effects controlling susceptibility to tumor development. Although these sophisticated tools have been extremely useful, the RCS methodology is very labor intensive and time-consuming, due to the need to generate, maintain, and phenotype a battery of RCSs. Only a very small number of strains have been developed into RCSs, and consequently the phenotypic and genetic variation accessible by this approach is, at present, limited (6). In addition, although the RCSs have been successfully used to detect genetic interactions (3, 7), the number of interactions that can be detected is limited by the segregation of the donor genome across multiple lines. Similar limitations apply to the analysis of interactions using a recently developed panel of chromosome substitution or "consomic" strains, in which single chromosomes from the donor background are segregated on a different host strain (8). We demonstrate here the feasibility of identifying significant and reproducible genetic interactions between tumor modifier loci in whole genome scans of interspecific *Mus musculus/Mus spretus* backcrosses.

Materials and Methods

Animals and Tumor Induction. Inbred *NIH/Ola* mice were purchased from Harlan Olac. Outbred *Mus spretus* and inbred *SEG/Pas* mice (derived from *Mus spretus)* were obtained from Drs. S. Brown (Medical Research Council, Harwell, England) and Jean-Louis Guenet (Institut Pasteur, Paris, France), respectively. SPRET/*Ei* mice were obtained from The Jackson Laboratory (Bar Harbor, ME). In NSP, NSE, and NSJ crosses, the same breeding and tumor induction protocols were carried out, and papilloma susceptibility was estimated by the number of papillomas at 20 weeks after initiation, as reported previously (1). 106 NSE and 162 NSJ animals were used for assessment of skin papilloma susceptibility, and the phenotype data on 326 NSP animals were reported previously (1).

DNA Preparation and Genotyping Using Microsatellite Markers. DNAs were prepared from tails and amplified by standard methods. One hundred six informative NSE mice were genotyped at 43 markers where evidence of linkage to skin tumor susceptibility in the NSP backcross was detected. The complete set of markers used for the NSP backcross were reported elsewhere (2). NSJ backcross mice were genotyped at markers on chromosomes 7 and 12 that were shown to exhibit strong interactions in the other two crosses.

Linkage Analysis. Tumor multiplicity in chemically induced mouse tumor experiments frequently follows a binomial negative distribution (9, Fig. 1), which is a generalized Poisson distribution, especially when the tumor number is overdispersed. Therefore, we used a negative binomial regression analysis to screen for predisposition loci and to identify interacting loci. Regression analysis has been shown previously to improve the power in detecting QTLs in plants and to be effective in minimizing the contribution of background genetic effects of segregating QTLs that could confound detection of epistatic interactions (10).

Interaction terms among all combinations of two loci were estimated by multiplying the values assigned to the mice on the basis of the marker

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[3] The abbreviations used are: QTL, quantitative trait locus; RCS, recombinant congenic strain; NSP, (*NIH/Ola* × outbred *Mus spretus*) × *NIH/Ola*; NSE, (*NIH/Ola* × inbred *SEG/Pas*) × *NIH/Ola*; NSJ, (*NIH/Ola* × inbred *SPRET/Ei*) × *NIH/Ola*; LOD, logarithm of odds.

genotypes. In our study, the number of papillomas (Y) is found to follow a negative binomial distribution:

$$Pr\{n = N\} = \frac{\Gamma(N+\theta)}{\Gamma(N+1)\Gamma(\theta)}\left[\frac{\theta}{\theta+\mu(x_i)}\right]^{\theta}\left[\frac{\mu(x_i)}{\theta+\mu(x_i)}\right]^{N} \quad \text{(A)}$$

Where θ indicates the heterogeneity of response,

$$\mu(x) = e^{\{\Sigma_i a_i x_i + \Sigma_i \Sigma_j b_{ij} x_i * x_j\}}$$

is mean, and x_i is the value of marker I. x_i is 1, if marker I is homozygous. x_i is 0, if marker I is heterozygous. Thus, all analyses are carried out in a multistage stepwise fashion using negative binomial regression. When the interaction or the locus had a $P < 0.01$, this interaction or the locus was kept in the model. When the locus or the interaction had a $P > 0.01$, this interaction or the locus was removed from the model and then the next variable was added and the model retested. The data set for carcinoma incidence, which is a dichotomous trait, was analyzed by a logistic model (1). If the interacting loci show strong evidence of linkage as single QTLs with major effects, the epistatic effect can be detected by any appropriate test. If, on the other hand, interactive loci do not show evidence of linkage as single QTLs, the threshold needs to be raised substantially. We have, thus, calculated the empirical suggestive and significant *P*s for interaction in the model by 5000 runs of simulation using random ordering of the same data set of phenotypes by Monte Carlo sampling (7). For the NSP cross, the *P* for suggestive linkage to papilloma development was 6.2×10^{-5} (LOD score, 3.4) and for significant linkage was 6.7×10^{-6} (LOD score, 4.4); for suggestive linkage to carcinoma development the equivalent value was 6.15×10^{-4}, and for significant linkage it was 5.73×10^{-5}. Criteria for significant and suggestive linkage for single markers are taken from Lander and Kruglyak (11).

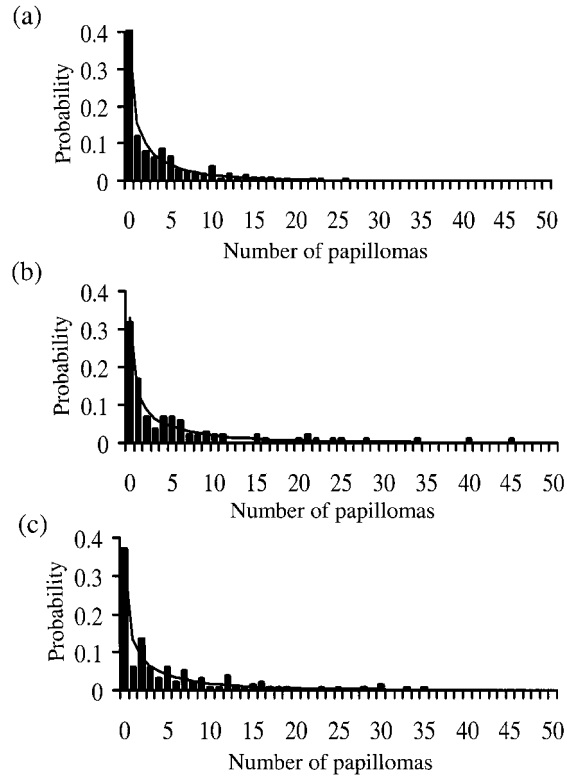

Fig. 1. Distribution patterns of papilloma multiplicity data in NSP, NSE, and NSJ. The number of skin papillomas at 20 weeks after initial treatment in 326 NSP (*a*), 106 NSE (*b*), and 162 NSJ (*c*) animals are plotted as histograms. Negative binomial distribution is depicted on the histogram as a *solid line*.

Results and Discussion

Table 1 shows a summary of the results of whole genome scanning for all possible combinations of pairwise interactions, using the negative binomial regression method. Significant evidence of linkage to papilloma multiplicity in the NSP backcross was obtained for interactions between *Skts1* (chromosome 7) and *Skts5* (chromosome12), *Skts7* (chromosome 4) and *Skts3* (chromosome 5), and *Skts9* (chromosome 16) and *D15Mit6*. The strongest interaction (LOD, 20.65) was found between the *Skts1* and *Skts5* loci on chromosomes 7 and 12, respectively. Animals heterozygous for the *D7Mit87* markers or for the *D12Mit154* markers had relatively low papilloma numbers, as did the double heterozygotes (Table 1 and Fig. 2*a*), but the absence of *spretus* alleles at both loci was associated with a substantial increase in papilloma number. This suggests that *spretus* alleles at these loci have redundant effects in conferring the resistance phenotype and may affect the same biochemical pathway in controlling papilloma number.

In addition to the significant interactions shown in Table 1, a number of other loci were involved in multiple interaction. For example, *Skts6* (chromosome 9) was involved in interaction with both *Skts10* (chromosome 17; $P = 1.3 \times 10^{-4}$) and *Skts2* (chromosome 7; $P = 9.0 \times 10^{-4}$). Three-way interaction *P* is 1.2×10^{-6}.

All of the markers involved in the significant interactions had previously been detected as single locus QTLs by negative binomial regression analysis (2), with the exception of the *D15Mit6* marker, which was only detected by virtue of its interaction with *Skts9*. Homozygosity of the *musculus* allele at *Skts9* increased the average papilloma number from 2.2 to 5.0 with respect to the corresponding heterozygous state, but this effect was only detected in the background of heterozygosity at the *D15Mit6* marker on chromosome 15. The main effects of QTLs can be masked by interactions that may change with genetic background or in response to environmental factors (12). Tumor modifier loci that only reached significance through interactions with other genomic loci have been detected previously using the RCSs (3, 4, 7).

Table 1 *Pairwise interactions for papilloma multiplicity data in the NSP cross*

Significant linkage $P < 6.7 \times 10^{-6}$ for interacting locus simulations using the multistage stepwise regression method.

Interaction	Genotype		Negative binomial regression method		
Genotype	Average papilloma no. (no. of mice)		LOD score	Coefficient (SE)	*P*
Skts1 × *Skts5*	*Skts5 (D12Mit154)*				
Skts1 (D7Mit87)	ns	nn			
ns	1.7 (88)	2.8 (82)			
nn	2.1 (70)	6.0 (86)	20.65	1.2202 (0.1397)	$<10^{-16}$
Skts7 × *Skts3*	*Skts3 (D5Mit77)*				
Skts7 (D4Mit37)	ns	nn			
ns	1.9 (74)	2.3 (79)			
nn	3.3 (88)	5.1 (85)	8.73	0.8481 (0.1337)	2.2×10^{-10}
Spr9 × *D15Mit6*	*D15Mit6*				
Spr9 (D16Mit2)	ns	nn			
ns	2.2 (80)	2.4 (88)			
nn	5.0 (84)	3.2 (74)	4.63	0.6707 (0.1453)	3.9×10^{-6}

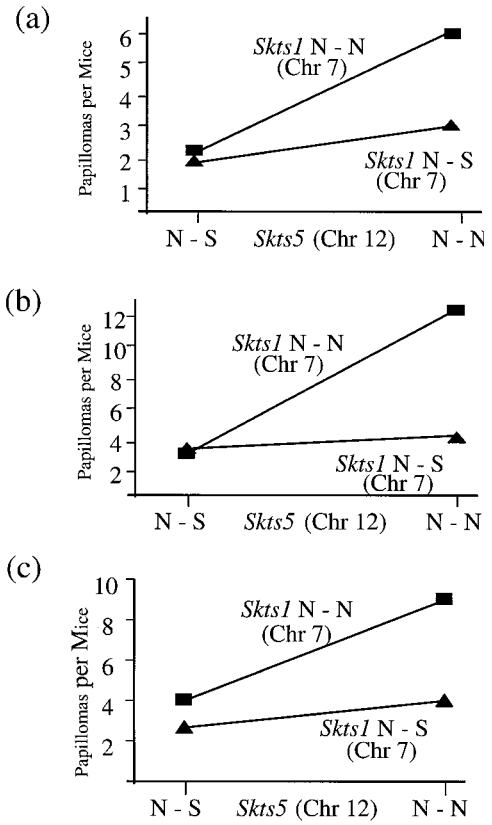

Fig. 2. Genetic interaction between *Skts1* and *Skts5*; the average papilloma numbers of four genotypes: Homozygous-Homozygous, Homozygous-Heterozygous, Heterozygous-Homozygous, and Heterozygous-Heterozygous, at *Skts1* and *Skts5* on chromosomes 7 and 12, are plotted. *a*, the interaction in NSP cross. *b*, the interaction in NSE cross. *c*, the interaction in NSJ cross.

One significant interaction [between *Skts3* (chromosome 5) and *Skts11* (chromosome 6)] affected carcinoma incidence ($P = 8.5 \times 10^{-7}$). One suggestive interaction was found between *Skts3* and the chromosome 15 marker *D15Mit13* ($P = 4.2 \times 10^{-4}$), which is 7.0 cM distant from the *D15Mit6* marker mentioned above. Again, this suggestive linkage was only detected through interaction with *Skts3* and not as a single locus.

The most compelling evidence in favor of epistatic interactions is obtained by independent replication of the same result in another cross. We, therefore, sought to confirm the interaction between *Skts1* and *Skts5* by carrying out an independent experiment involving an inbred line of *Mus spretus* (*SEG/Pas*) crossed to *NIH/Ola*. The *SEG/Pas* mice were originally derived from European *Mus spretus* but have been inbred for more than 20 generations. To confirm the resistance phenotype of *SEG/Pas*, we performed two-stage skin carcinogenesis with 5 *SEG*, 6 *NIH/Ola*, 11 (*SEG/Pas* × *NIH/Ola*) F1, and 106 NSE F1 backcross animals. No skin papillomas were seen 20 weeks after initiation of treatment of *SEG/Pas* mice and the F1 hybrids, apart from one F1 animal with a very small skin papilloma. In contrast, all of the *NIH/Ola* mice had many skin papillomas (average number, 21). NSE backcross animals had an intermediate phenotype. A χ^2 test showed a good fit of tumor multiplicity data to a negative binomial distribution (13), as shown previously for the complete NSP backcross (2). Because our papilloma data conform to a negative binomial distribution ($P = 0.10$ for NSP, 0.18 for NSE, and 0.2 for NSJ, Fig. 1), the following statistical analyses were all based on this distribution.

Interestingly, the only significant *SEG/Pas* papilloma resistance locus was detected at the marker *D1Mit102* on chromosome 1 ($P = 2.1 \times 10^{-6}$), only 10 cM centromeric from the *Skts8* locus found in the previous NSP backcross (2). The previously detected loci *Skts1* and *Skts5* on chromosomes 7 and 12, respectively [at markers *D7Mit246* on chromosome7 (12 cM centromeric from *Skts1*) and *D12Mit182* on chromosome12 (15 cM centromeric from *Skts5*)], were also found in the cross, but these only reached suggestive significance ($P = 5.0 \times 10^{-4}$ and $P = 3.4 \times 10^{-4}$, respectively). Despite this failure to reach significance as independent QTLs in this second experiment with inbred *spretus* mice, the previously detected interaction between the loci on chromosomes 7 and 12 was significant [interaction between *D7Mit246* and *D12Mit182* ($P = 5.6 \times 10^{-12}$)]. The absence of inbred *spretus* alleles at both markers increased the average papilloma number from 3.6 to 12.5, whereas no significant effect was seen in animals that retained one *spretus* allele at either location (Fig. 2*b*). This confirms in a completely independent study the robustness of this interaction, which is detectable even in experiments involving relatively small numbers of animals. A similar interaction was also found in a third backcross carried out involving *SPRET/Ei* mice, independently derived inbred strain of *Mus spretus* obtained from The Jackson Laboratory. In this cross, the average papilloma number increased from 3.06 in mice heterozygous at the markers *D7Mit198* and *D12Mit153* on chromosomes 7 and 12, to about 9.40 papillomas per mouse in the homozygotes (Fig. 2*c*). The *P* for the interaction in this cross was 2.0×10^{-4}. When the results of the three experiments were combined, the total combined LOD score was 29.26 for this interaction. To our knowledge, no other genetic interactions between tumor modifier loci have been confirmed in independent crosses.

The approach we have described may be amenable to detection of interactions between tumor modifier loci in crosses between any strain of *Mus musculus* that is susceptible to tumor development, and a wild mouse species such as *Mus spretus.* One potential disadvantage of using interspecific crosses is that the large number of genetic differences between *Mus musculus* and *Mus spretus* may complicate the ultimate identification of the critical polymorphisms responsible for the modifier effect. For this purpose, the comparison between outbred *Mus spretus* and the two inbred strains of *Mus spretus* that we have used in this study may be particularly useful, because the smaller number of genetic differences will facilitate the detection of critical functional alterations.

Another issue concerns the use of backcrosses or intercrosses for detection of genetic interactions. Although the use of an interspecific backcross may allow us to detect only some of the epistatic effects, it can also reduce the complexity, because of lower statistical freedom and reduction of genetic variance caused by gene interactions (14). If extreme phenotypic differences exist between two strains or species and dominant interactions are expected, the interaction components can be detected more efficiently using a simple backcross (14). The approach we have described obviates the necessity to transfer transgenes, knockouts, or susceptibility loci on to genetic backgrounds suitable for analysis using the relatively limited range strains for which recombinant congenic lines are available.

Present attempts to detect tumor modifiers in human populations are limited to association studies involving candidate genes. Our ability to detect significant associations in human populations will clearly be affected by the presence of interacting genes that, dependent on genetic background, can make a particular locus "invisible" or substantially more difficult to find. It is possible that the identification of interacting pathways between tumor modifier loci using model organisms such as the mouse will simplify this search by guiding the choice of candidates for association studies.

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