Genetic Analysis of Three-dimensional Shape of Mouse Lung Tumors Reveals Eight Lung Tumor Shape-Determining (Ltsd) Loci That Are Associated with Tumor Heterogeneity and Symmetry

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ABSTRACT

Most lung tumor linkage studies focus on identifying loci that confer susceptibility or resistance irrespective of the tumor types developed. However, different mouse strains develop different types of lung tumors. A major obstacle for genetic studies of these differences is the lack of reproducible, quantitative, and uniform assessment of tumor type. We have previously described a new variable (Rratio) that assesses the three-dimensional shape of lung tumors in a quantifiable way and showed that nonspherical tumors are correlated with tumor heterogeneity and with a tendency to asymmetrical growth. We tested the F2 cross between the susceptible strain O20 and the recombinant congenic strain OcB-9. Both develop mixed alveolar and papillary lung tumors, and the OxB9 tumors are, on average, more elongated than the O20 ones. We mapped eight new lung tumor shape-determining loci (Ltsd1–8) involved in mutual interactions. Two of these loci, Ltsd1 and Ltsd3, seem to play a major role in tumor shape formation. The Ltsd4 locus was confirmed in a second F2 cross between strain O20 and the recombinant congenic strain OcB-6. Genotype-phenotype associations show that nonspherical tumors are correlated with tumor heterogeneity and nonsymmetrical (focal) development of structures. Most of the new Ltsd loci map in regions where susceptibility to lung cancer (Sluc) loci have been previously mapped, raising the question of whether they are identical or closely linked loci. Based on models of tumor growth indicating that supply of nutrients and the ability to create a capillary network may be shape-determining factors, we suggest as likely candidates for the Ltsd loci genes involved in angiogenesis, vascularization, and capillary patterning. This is the first set of loci that affects qualitative aspects of lung tumors and may provide biologically and clinically interesting indicators of tumor progression.

INTRODUCTION

Cancer arises from an accumulation of mutations that promote clonal selection of cells with increasingly aggressive behavior. Lung cancer is the most frequent cancer worldwide, accounting for most cancer deaths (first among men and third among women; Ref. 1). The microscopic appearance of human cancer has been for most of this century the major predictor of its clinical behavior. In tumor pathology, detailed description and analysis of the cellular and subcellular particularities that are associated with various cancers and tumor types are used to categorize. More recently, with the advent of DNA array technology, complex molecular profiles based on expression patterns of hundreds or thousands of genes are built that correlate with already known tumor types/classes and suggest the existence of new (2–4). As new tumor subclasses are described through such technologies, there is a need for new morphological descriptors and for qualitative assessment of tumor types that will match and validate the emerging expression clusters.

Most histological classifications of tumors are derived from two-dimensional surfaces, and few studies have attempted to model and measure epithelia in three-dimensional space (5–7). Three-dimensional shape analysis has been used to classify and help in the early detection of human melanomas (8, 9), whereas attempts to visualize and evaluate three-dimensional shape in pulmonary lesions have been less successful (10). We have recently described a new method for qualitative assessment of mouse lung tumors that provides a numerical description of the three-dimensional shape of a tumor. Analysis of lung tumors derived from various inbred and RC4 strains has shown that the new three-dimensional shape variable, called Rratio, is correlated with tumor heterogeneity and tumor asymmetry. One of the obvious advantages of using a quantifiable variable to assess the histological structure of a tumor is that it can be used in statistical tests, including linkage analysis. We took advantage of this by applying the Rratio measurement on a well-characterized set of tumors derived from a F2 cross between RC strain O20/B and strain O20. This cross has been extremely informative in the past in mapping susceptibility to lung cancer (Sluc) loci (12–14).

The RC O20 strains (O20-congenic-B10.O20/Dem) were constructed using as donor strain the lung tumor-resistant B10.O20/Dem (referred to henceforth as B10.O20) strain and as background strain the lung tumor-susceptible O20/A (referred to henceforth as O20) strain (15, 16). Each RC strain carries a random 12.5% of the genome derived from the donor strain. Strain B10.O20 develops papillary nonheterogeneous tumors, with usually well demarked borders and little or no nuclear pleiomorphism, whereas strain O20 develops both alveolar and mixed alveolar and papillary tumors with varying degrees of tumor heterogeneity and with localized or general nuclear pleiomorphism (17). The O20 lung tumors are also mixed alveolar and papillary tumors, usually heterogeneous, and with nuclear pleiomorphism. They are less spherical but not significantly different from the O20 strain tumors. The (O20-9xO20)(O20-9xO20)F2-derived tumors are significantly less spherical than the O20 tumors (11). Detailed analysis of spherical and nonspherical tumors in this cross has revealed strong association of nonspherical shape with tumor heterogeneity. In addition, when sorting tumors according to their shape, the association between tumor heterogeneity and nuclear pleiomorphism is significantly high in nonspherical tumors but not in spherical tumors (11). Here we report the mapping of shape-determining loci in the (O20-9xO20)(O20-9xO20)F2 cross and how the mapping of such loci enables the detailed histological classification of mouse lung tumors.

MATERIALS AND METHODS

Mice and Carcinogen Treatment. The strain O20/A, the H2 congenic strain B10.O20/Dem, and the O20-congenic-B10.O20/Dem (OcB) series of

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MOUSE Ltsd LOCI CORRELATE WITH TUMOR HETEROGENEITY

Fig. 1. A, schematic representation of heterogeneous asymmetrical tumor showing focal patterns of alveolar (pink) and papillary (red) growth. The box represents the approximate place that is shown in the photo below, showing clear papillary features and more intensely stained cells on the left, and alveolar structures with higher nuclear pleomorphism on the right (×200 magnification). B, schematic representation of heterogeneous symmetrical tumor with peripheral (pink) alveolar and internal secondary papillary (red) structures. The box represents the approximate place that is shown in the photo below, showing alveolar adenoma structures, whereas further in, typical papillary projections can be seen (×200 magnification).

RESULTS

Eight new loci have been detected that influence the overall three-dimensional shape of a tumor, and Table 1 summarizes the linkage results. We termed the new loci lung tumor shape-determining (Ltsd) loci.
is also homozygous for the B10.O20 allele, whereas when Ltsd1 is associated with nonspherical tumors only when the interacting locus
squares means of the SAS output. The average Rratio of the cross is 17.5%.
(higher the value, the more elongated the tumor) and is back-transformed from least
of tumors is more spherical than the cross average (Table 1). Exactly
(homozygous for the O20 allele), the average three-dimensional shape
becomes visible when the interacting locus is taken into account. For
individual effect is masked by the effect of the interacting locus but
loci.

**Table 1** Detection of eight Ltsd loci in the (OcB-9xO20) F2 cross

<table>
<thead>
<tr>
<th>Ltsd1 (D18Mit17)</th>
<th>25.4% ± 0.3520</th>
<th>17% ± 0.0564</th>
<th>14% ± 0.0564</th>
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<tr>
<td>bo</td>
<td>23% ± 0.00627</td>
<td>15% ± 0.0335</td>
<td>23% ± 0.0335</td>
</tr>
<tr>
<td>oo</td>
<td>15% ± 0.00627</td>
<td>17% ± 0.0335</td>
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B. Ltsd4 (D4Mit158)

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<th>Ltsd4 (D8Mit3)</th>
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<td>bo</td>
<td>22% ± 0.00627</td>
<td>16% ± 0.0335</td>
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<td>oo</td>
<td>15% ± 0.00627</td>
<td>22% ± 0.0335</td>
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C. Ltsd5 (D7Nds2)

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<tr>
<th>Ltsd5 (D7Nds2)</th>
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<th>15% ± 0.0335</th>
<th>17% ± 0.0335</th>
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<tbody>
<tr>
<td>bo</td>
<td>17% ± 0.00627</td>
<td>25% ± 0.0335</td>
<td>19% ± 0.0335</td>
</tr>
<tr>
<td>oo</td>
<td>12% ± 0.00627</td>
<td>18% ± 0.0335</td>
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D. Ltsd3 (D8Mit3)

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<tr>
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<td>19% ± 0.0335</td>
</tr>
<tr>
<td>oo</td>
<td>12% ± 0.00627</td>
<td>18% ± 0.0335</td>
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E. Ltsd7 (D16Mit9)

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<td>17% ± 0.0335</td>
<td>17% ± 0.0335</td>
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<tr>
<td>oo</td>
<td>14% ± 0.00627</td>
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F. Ltsd8 (D6Mit218)

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<td>18% ± 0.0335</td>
<td>20% ± 0.0335</td>
</tr>
<tr>
<td>oo</td>
<td>13% ± 0.00627</td>
<td>14% ± 0.0335</td>
<td>24% ± 0.0335</td>
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</table>

Homozygous for the B10.O20 allele.  4 Homozygous for the O20 allele.
5 Phenotype (Ratio) is given as a percentage difference from a perfect sphere (the higher the value, the more elongated the tumor) and is back-transformed from least squares means of the SAS output. The average Ratio of the cross is 17.5%.

the opposite happens for Ltsd2α/β, where the most spherical tumors are associated with the Ltsd2α/β genotype.

The effects of the Ltsd6 locus are sex dependent. The Ltsd6α/β genotype is associated with nonspherical tumors in female mice and with spherical tumors in male mice, whereas the opposite effect of sex is observed with the Ltsd6α/β genotype. The estimated main effects of Ltsd6α/β and Ltsd8α/β are only −0.01 and 0.038, respectively. Similarly low is the estimated main effect of sex (0.17 for female and 0.006 for male). However, the interaction between sex and Ltsd8 explained 5.49% of variance (Table 1).

In Table 1, the explained variance for each interaction is given. It ranges from 4% (for the Ltsd7×Ltsd6 interaction) to 7.85% (for the most significant interaction Ltsd1×Ltsd7). The total explained variance in the (OcB-9xO20)F2 cross, using the final model that yielded these significant linkages, is 37.76%.

The presence of B10.O20-derived segregating segments containing Ltsd loci in more than one OcB strain presented us with the opportunity to use them for confirmation of these Ltsd loci. The length of the chromosomal region where Ltsd was mapped in the OcB-9 F2 cross is 13 cM [minimal (see Fig. 2 for definition) to 24.5 cM (maximal; Table 2; Fig. 2) and a centromeric part of this region is also present in the OcB-6 strain (between markers D4Mit66 and D4Mit42; Fig. 2), and a centromeric part of this region is present in the OcB-16 strain (between markers D4Mit37 and D4Mit158; Fig. 2). When we tested the OcB-16 F2 cross for confirmation of the Ltsd4 locus, no significant linkage was detected, whereas in the OcB-6 F2 cross, a significant linkage was detected with the D4Mit66 marker (point-wise P = 0.02). The results from the three crosses not only confirm the presence of a lung tumor qualitative locus in the region but also likely exclude the centromeric part of the original candidate region (between markers D4Mit37 and D4Mit66; Fig. 2). Thus, the size of the candidate region is reduced to 2 cM (minimal) to 12 cM (maximal).

We have proposed in the past that linkage analysis with the Rratio shape variable will point to specific subsets of tumors based on the various genotypic and phenotypic combinations that can be further analyzed microscopically (11). We therefore analyzed an approximately equal number of tumors from three genotypic combinations of the most significant interaction, that between Ltsd1 and Ltsd7, and compared the results. Two genotypic combinations are associated with more spherical tumors (Ltsd1α/βLtsd7α/β and Ltsd1α/βLtsd7β/β; Table 1), and one is associated with more elongated tumors (Ltsd1α/βLtsd7α/β; Table 1). In each case, tumors were sorted according to Rratio, and approximately 10 most spherical and 10 most elongated (nonspherical) tumors were analyzed. We compared the presence of different phenotypic features in tumors derived from mice of different genotypes and in spherical and nonspherical tumors. Table 3 summarizes these results. Originally, by evaluating the tumors of the OcB-9 F2 cross, we found that heterogeneous tumors were more often nonspherical than nonheterogeneous and vice versa (P = 0.0335; Ref. 11). We show here that the same association is significant only when both Ltsd1 and Ltsd7 are homozygous for the B10.O20 allele (Table 3; 11 of 12 spherical tumors are nonheterogeneous, and 8 of 9 nonspherical tumors are heterogeneous; Fisher’s exact test, \( P = 3.67 \times 10^{-4}, P = 0.0033 \). Interestingly, this is not the case for the other two genotypic combinations. Comparison of the heterogeneity status of nonspherical tumors from all three genotypic combinations indicates a difference between the Ltsd1α/βLtsd7α/β and Ltsd1α/βLtsd7β/β tumors (G-test, 10.1436, 2 degrees of freedom, \( P = 0.00627, P = 0.0564 \). This may be partly explained by the difference in symmetry of these tumors (5 of 9 versus 10 of the two genotypes).

Another indication of a close association of shape with tumor...
heterogeneity can be found when looking for linkage between the tested markers and shape, using only the analyzed tumors (145 in total). In essence, this constitutes an analysis of selected extreme phenotypes for shape. One would expect that if shape is associated with heterogeneity, then the correlation between some of the detected loci and shape would be influenced depending on the heterogeneity status of the tumors. This is shown in Table 4. The significance of tumor heterogeneity can be estimated by calculating the \( G^2 \) for heterogeneity, with the formula \( G^2 = G - G_w \), where \( G \) is the sum of the \( G \) tests for heterogeneous only and nonheterogeneous only tumors, and \( G_w \) is the \( G \) test for all tumors (20). In the case of \( Ltsd1 \), \( G \) = 11.9925 (2 degrees of freedom, \( P = 0.0025 \)), indicating a significant contribution of tumor heterogeneity to the linkage of \( Ltsd1 \) to tumor shape for heterogeneous only tumors, whereas for the \( Ltsd2 \) and \( Ltsd8 \) loci, the resulting \( G^2 \) values are nonsignificant (\( P > 0.05 \)).

In the past, we were able to demonstrate a suggestive correlation between tumor asymmetry (Fig. 1, A and B) and nonspherical tumors (using 105 tumors; Fisher’s exact test, \( P = 8.53 \times 10^{-3} \), \( P = 0.0938 \); Ref. 11). With the inclusion of 40 additional tumors in this study, the correlation becomes significant (21 nonsymmetrical tumors of 75 nonspherical tumors versus 3 of 70 spherical tumors; Fisher’s exact test, \( P = 7.18 \times 10^{-5} \), \( P = 6.47 \times 10^{-4} \)). However, due to the overall small number of nonsymmetrical tumors, it is not possible to derive any useful phenotype-genotype associations for the \( Ltsd1 \times Ltsd7 \) interaction (Table 3). We then performed linkage analysis on the analyzed tumors for symmetry. There was no significant (after correction for the 13 tested markers and sex) linkage (main effects only) for any tested marker. However, when taking into account only nonspherical and heterogeneous tumors (37 tumors), the association between symmetry and \( Ltsd4 \) is suggestive (Table 5; \( G \)-test, 9.2925, 2 degrees of freedom, \( P = 0.0096 \), \( P = 0.1344 \)). The homozygous \( Ltsd4 \) O20 genotype is associated with nonsymmetrical (focal) tumors (9 of 10), whereas the homozygous B10.O20 genotype is associated with symmetrical tumors (8 of 11), suggesting a symmetry (focal development) determining locus.

### DISCUSSION

We have previously described a shape variable, Rratio, that provides a numerical description of the three-dimensional shape of a tumor, and we have demonstrated that although the Rratio variable does not discriminate between the commonly used classifications of alveolar and papillary tumors, it provides a measure of tumor heterogeneity and lack of symmetrical progression in all three dimensions.
applied the Rratio variable in the (OcB-9xO20)F2 cross because the to study the genetics of susceptibility to lung cancer. QTL analyses of of tumor shape. These crosses have been successfully used in the past ized segregating crosses of various OcB strains to study the genetics in studies of colon cancer susceptibility (23, 24), skin cancer suscep-

Ltsd

The presence of two-way interactions is recurring in both Ltsd (11). One of the obvious advantages of a numerical descriptor of shape is that it can be used for QTL analysis in a segregating cross. Identification of shape loci using morphometrics to describe bristles and wing shape in Drosophila is a good example (21, 22).

In the present study, we take advantage of already well-character-

ized segregating crosses of various OcB strains to study the genetics of tumor shape. These crosses have been successfully used in the past to study the genetics of susceptibility to lung cancer. QTL analyses of tumor size and tumor number revealed 30 Sluc loci (12–14). We applied the Rratio variable in the (OcB-9xO20)F2 cross because the average tumor shape is significantly more elongated than the average tumor shape of the parental strain O20 (11).

We identified eight loci linked with the shape of the tumor, which we termed Ltsd loci, and all are involved in two-way interactions (Table 1). The presence of two-way interactions is recurring in both lung tumor size and lung tumor number loci (12–14) and is also found in studies of colon cancer susceptibility (23, 24), skin cancer susceptibility (25, 26), and polycystic kidney disease (27) in mice; susceptibility to breast cancer in rats (28); and susceptibility to Leishmania major infection in mice (29, 30). The number of interactions currently identified is an indication of the actual level of complexity underlying genetic modifiers. In fact, taking into account that we are currently investigating only two-way interactions, due to the limited size of the test crosses, we are possibly only observing the “tip of the iceberg” of the actual interactions. The RCS system seems to be particularly advantageous in unveiling two-way interactions. Given that in any given RC cross only one-1/8 of the genome is segregating, the chance that both interacting loci segregate is 1 in 64. One would therefore expect that if two-way interactions were a rarity, they would be very infrequent in RC crosses. Projecting the current number of found two-way interactions to the total genome would suggest a large number of two-way interactions. In bristle shape analyses in Drosophila, strong epistatic interactions were evident in selection lines, suggesting a high frequency of interacting QTLs (31). However, it is also possible that higher order interactions exist, as suggested for cancer (14, 26), infection (29, 30), hypertension (32), asthma (33), and neurodegenerative diseases (21, 34), and that the two-way interactions we actually observe in the RC system are reflections of such higher order interactions.

Ltsd1 and Ltsd3 are possible major modifiers of tumor shape. For example, the Ltsd1 homozygous genotype is always associated with nonspherical tumors when the interacting loci (Ltsd2, Ltsd5, and Ltsd7; Table 1) are also homozygous for the O20 allele, and it is always associated with spherical tumors when the interacting loci are homozygous for the B10.O20 allele. In fact, there seems to be a general trend for nonspherical tumors when the interacting loci are both homozygous for the O20 allele or the B10.O20 allele, whereas when one is homozygous for O20 and the other for B10.O20, the resulting shape is usually spherical. Therefore, there are no per se spherical or nonspherical...
alleles, and tumor shape is, to a certain extent, the result of interplay between a number of genetic factors.

An interesting feature of most of the newly identified loci is that their chromosomal locations overlap with already mapped Sluc loci (Table 2 and Refs. 12–14). We have also previously shown that Sluc1 interacts with Sluc20 (14), and because the same regions harbor the interacting Ltsd3 and Ltsd6, we investigated the possibility of a combined size-shape effect, because Bookstein (35) suggests the possibility of size as a covariant of landmark data in morphometrics. However, there is no correlation between shape and size both for the entire cross and for the specific genotypic combinations that arise from these interactions (data not shown). It should be noted that the average candidate region length for both the Sluc and the Ltsd loci is approximately 15 cM, and therefore it is possible that there are two distinct sets of genes, one responsible for susceptibility, and the other responsible for shape. A potentially exciting theory would have quantitative and qualitative genes mapping close to each other, thus forming type-specific genetic niches.

The important question is what biological processes can determine or affect tumor shape and in what way. It has been suggested that a limiting nutrient may be a major determinant of tumor shape (36). In a nonlinear model of cancer growth, iron was used as the limiting factor, and different kinds of spheroids were observed by slightly modifying the ratio supply/affinity of iron. In the model, no tissue heterogeneity (inhomogeneity) that could result in position-dependent diffusivity was taken into account (36). Supply of nutrients in the proliferating neoplastic cells comes initially from the organ microenvironment by diffusion, but once the tumor exceeds a certain mass, neovascularization is necessary, and a capillary network is usually established from the surrounding host tissue (37). Interestingly, looking through the list of known mapped genes in the Ltsd candidate regions, a number of genes relevant to angiogenesis are found. For example, the fibroblast growth factor Fgf1 gene (38) maps in the Ltsd1 region, and Fgf6 (39) maps in the Ltsd8 region. The fibroblast activation protein Fap (40) and Vegf1 (41), a gene that correlates temporally and spatially with early differentiation of angioblasts, map in the Ltsd2 region, whereas another gene potentially involved in the mechanism of vascular development, Agrp2 (42), and the fibroblast growth factor receptor Fgfr1 map in the Ltsd3 region. The Ltsd4 and Ltsd5 regions harbor interleukins 14 and 16, respectively, and in the Ltsd4 region, Ephb2 (43), a member of the Ephb receptors involved in the regulation and formation of the vascular network, is also found. Obviously, these are candidate genes not only because of their function but also because of their known location, and even if they turn out not to be the actual Ltsd loci, the functional relationship between nutrient supply, angiogenesis, vascularization, and tumor shape may still be valid. In fact, increased microvesSEL density and measurement of angiogenesis index seem to be a significant independent prognostic indicator in cases of breast cancer (44), prostate cancer (45), melanoma (46), and colon (47) cancer.

The association between tumor shape and tumor heterogeneity is significant (Ref. 11 and this study), and here we show that the association between shape and tumor symmetry improves from suggestive ($P_r = 0.0038$ in Ref. 11) to significant ($P_r = 6.47 \times 10^{-4}$ in this study). In addition the shape-tumor heterogeneity association is evident and becomes significant when comparing specific genotypic combinations of interacting Ltsd loci (Table 3). Direct association between Ltsd loci and shape seems to be influenced by the heterogeneity status of the tumors, as seen for the Ltsd1 locus, but not for the Ltsd2 and Ltsd8 loci (Table 4). This is in general agreement with other efforts to study tumors in three dimensions and establish a positive correlation between structural atypia and grade of differentiation in glandular tumors of the stomach (5) and as a discriminatory tool for tubular and villous sectors of human tubulo-villous colorectal adenomas (48). Our set of heterogeneous tumors can be divided into two groups based on symmetry. The tumors that showed focal growth patterns and therefore have patches of atypia were characterized as asymmetrical (Fig. 1A). Interestingly, however, a large number of heterogeneous tumors seem to develop in a symmetrical concentric bizonal way. The outer zone is mostly alveolar and often exhibits a high degree of nuclear pleiomorphism and is more atypical than the internal zone, where secondary papillary structures with little or no nuclear pleiomorphism are seen (Fig. 1B). The need for blood supply in the central parts of such tumors may favor the secondary papillary formations that can recruit fibroblasts, thus creating this bizonal appearance. Expression profiling and DNA analysis of various zonal parts of such heterogeneous tumors could shed light on the sequence of events of tumor progression. Interestingly, linkage analysis between the tested markers and symmetry shows that upon selecting for elongated and heterogeneous tumors, there is a suggestive association between symmetry and the homozygote genotypes (O20 versus B10.O2O2-derived) of the Ltsd4 locus, implying a genetic control of focal development (Table 5).

The shape effects seen here are not always explained by differences in tumor heterogeneity or symmetry. This suggests that additional factors contribute to the determination of tumor shape. This study provides a first genetic step toward a molecular understanding of tumor shaping and progression. Ultimately, the combined efforts of mapping susceptibility and QTL will reveal whether tumor formation and tumor progression are regulated by closely associated pathways or whether the two events are mostly independent. Especially in the first case scenario, detection of QTL will provide valuable molecular markers for accurate diagnosis and possibly the design of more efficient tumor type-specific treatments.

Acknowledgments

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References

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