Predisposition to Efficient Mammary Tumor Metastatic Progression Is Linked to the Breast Cancer Metastasis Suppressor Gene Brms1

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ABSTRACT

Tumor metastasis is one of the most important clinical aspects of neoplastic disease because patient mortality is frequently attributable to disseminated rather than primary tumors. However, it still is not possible to definitively distinguish those individuals at high risk for disseminated disease, who would benefit from aggressive adjuvant therapy, from the low-risk patients who might be spared the side effects of additional anticancer therapy. To identify factors that predispose toward metastatic disease, we have used a genetic approach. Using a highly metastatic model of mammary cancer, we identified previously inbred mouse strains (DBA/2J, NZB/B1NJ, and I/Lnj) that harbor genetic factors that significantly suppress metastatic efficiency. In this study, we report the results of four experiments to localize the genetic map locations of the metastasis efficiency modifier genes. One statistically significant locus was identified on proximal Chr 19 designated Mies1. Secondary candidate intervals were detected on Chrs 6, 9, 13, and 17. Interestingly, Mies1 colocalizes with the murine orthologue of the human breast cancer metastasis suppressor gene Brms1, suggesting that allelic variants of Brms1 might be responsible for the metastasis suppression observed.

INTRODUCTION

The process of tumor dissemination or metastasis is an important aspect of clinical management of cancer. In most cases cancer patients with localized tumors have significantly better prognoses than those with disseminated tumors. The majority of cancer mortality has been associated with metastatic disease rather than the primary tumor (1). Because it has been estimated that 60–70% of patients have progressed to metastatic disease by the time of diagnosis (2), better understanding of the factors leading to tumor dissemination is of vital importance. The ability to identify those patients at high risk of metastatic disease may permit more aggressive therapy while sparing the low risk cohort the side effects of additional anticancer treatment.

The metastatic cascade is understandably complex, with many potential barriers. Successful tumor dissemination requires that tumor cells escape the primary tumor, invade the surrounding tissues, and are transported to secondary sites where they proliferate as secondary masses. Hematogenous metastases enter into the vascular or lymphatic system and, once in the circulatory system, the cells must arrest in the target tissue, escape out of the blood vessel, and penetrate the adjacent tissue. Finally, the tumor cells must be able to proliferate in the foreign microenvironment and initiate angiogenic recruitment of new vasculature to allow the disseminated tumor to grow beyond microscopic size (1). An enormous amount of research has been performed elucidating various components of this process. As a result a great deal is known about different molecules and pathways that are associated with metastatic progression, including activation of oncogenes (3, 4), recruitment of metalloproteases (5–8), and motility factors (9, 10). In addition, a number of chromosomal abnormalities have been associated with breast tumor dissemination in humans, including loss of Chrs 1, 1p, 3p, 6q, 7q, 11p, and 11q (11). Metastasis-associated loss of heterozygosity has been used as a tool to identify members of a class of genes known as the metastasis suppressors. Analogous to tumor suppressors, metastasis suppressors can be distinguished from the former in that they prevent tumor dissemination when introduced into cancer cells but do not affect tumor initiation (12).

Despite this wealth of information, the critical initiating events or molecular pathways for tumor dissemination remain unclear. Part of the difficulty unraveling the complexity of metastasis may be attributable to multiple pathways converging on the same phenotype. Another confounding factor is likely to be genetic susceptibility to metastatic progression or genetic modulation of the efficiency of tumor dissemination. The presence of genetic modulation has been demonstrated both in vitro and in vivo. Tumors resulting from the transfection of oncogenes into cell lines derived from different mouse inbred strains show dramatically different metastatic abilities without affecting primary tumor formation (3). This suggested the presence of metastatic suppressing alleles in the genetic background of one mouse strain compared with a second metastatic mouse strain. We have demonstrated previously a significant impact of genetic background on the initiation, progression, and metastatic dissemination of the poten polyoma middle-T mammary tumor model. Alterations in tumor latency, tumor growth rate, and metastatic efficiency were observed in progeny of the F1 generation of the PyMT bred to 27 different inbred strains (20). In the crosses analyzed to date it has been observed that the loci modulating the various phenotypes are genetically distinct (21, 22). Furthermore, different strain combinations can modify one, two, or three of the measured phenotypes, and, therefore, provide tools and reagents to specifically dissect the genetic components of various stages of mammary tumorigenesis.

Identification of key regulatory components of the metastatic process would serve two functions. First, they might provide more accurate prognostic markers of potential metastatic progression in patients than the current standards (23–32). Second, they may provide insights into the critical events in tumor dissemination, potentially leading to additional avenues of research or the development of novel therapies. Therefore, the current study describes the results of four different experiments to map the genetic components of efficient mammary tumor dissemination. Reproducible linkage to proximal Chr 19 was observed, linked to the breast cancer metastasis suppressor gene Brms1. Suggestive linkage to additional chromosomes was also uncovered.

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2 The abbreviations used are: Chr, chromosome; QTL, quantitative trait loci; PI3k, phosphatidylinositol 3’kinase.
MATERIALS AND METHODS

Animals. FVB/N-TgN(MMTVPyVT)634Mul mice were obtained from William Muller, McMaster University, Hamilton, Ontario, Canada (33). FVB/NJ, NZB/BINJ, DBA/2J, AKXD/TyJ, and I/LnJ mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. The generation and genotyping of the I/LnJ backcross was described in Le Voyer et al. (21). The backcross (N2) animals were generated by breeding FVB/N-TgN(MMTVPyVT)634Mul males to females of the other inbred strains (I/LnJ, DBA/2J, and NZB/BINJ), and the resulting transgene-positive F2 hybrids mated back to FVB/NJ females. Inheritance of the polyoma transgene was determined by PCR amplification of weanling tail biopsy DNA with the following primers: 5'-AAC GGC GGA GCG AGG AAC TG-3' and 5'-ATC GGG CTC AGC AAC ACA AGC-3'. The AKXD RI mapping experiment was performed by mating the FVB/N-TgN(MMTVPyVT)634Mul males to females of each of the AKXD recombinant inbred lines and analyzing the transgene-positive female F2 hybrids.

Determination of Metastatic Efficiency. Transgene-positive females were maintained at three to five animals per cage and screened by palpation three times a week for the presence of the primary mammary tumor. Diagnosis was performed by a single operator to minimize interpersonnel variance. The location of the tumor was recorded, and the animals were examined for an additional week to confirm diagnosis and then aged for 40 days after diagnosis to permit development of metastases. After 40 days, the animals were sacrificed by carbon dioxide inhalation and the lungs harvested for histological examination. The lung tissues were fixed in 10% paraformaldehyde, embedded in paraffin, sectioned, and stained with H&E.

Statistical Analysis. The large number of animals with a pulmonary metastatic density of zero precluded use of traditional QTL mapping tools, which assume normally distributed values (34). An alternative nonparametric approach, in which trait values are replaced with ranks, is also not ideal for our data, because many individuals have identical trait values (35). To overcome these problems, we analyzed the survival time data using a recently described single-QTL model (36). In this model, mice with QTL genotypes AA and AB have probability pA and pB, respectively, of having metastases. If a mouse does not have metastatic involvement, the log of the density of pulmonary metastases y is assumed to be normally distributed with mean μA or μB, according to its genotype, with a SD σ. To map the trait, we calculated three separate lod scores. The statistic lod(p) measures the probability of a mouse of a given genotype (pA or pB) having metastatic disease. The statistic lod(μ) measures differences in the average density of pulmonary metastases among those mice of a given genotype among those animals with metastatic involvement. The statistic lod(p, μ) is a combination of the lod(p) and lod(μ) scores and measures the combined efficiency of metastasis (the probability of having metastases as well as the density of metastatic lesions). In the AKXD RI lines, single-marker analyses were performed. In the three backcrosses, interval mapping was used. To additionally explore the genetic determinants of mammary tumor metastatic efficiency and to obtain confirmatory results (38), linkarge analysis of the cross revealed two loci at which the LOD scores were substantially above the empirically determined genomewide 5% threshold of significance (Fig. 2; Table 1). One of the key advantages of this RI backcross design is that the female F1 progeny are isogenic but recombinant. The genetic structure of the RI backcross progeny resembles a conventional N2 but with a much higher load of recombination breakpoints per genome. Therefore, the development and severity of tumor metastasis can be estimated accurately for each of the recombinant backcross progeny.

RESULTS

Previous studies demonstrated that the metastatic phenotype in F1 hybrids between the PyMT mouse and AKR/J mice was not significantly different from that of the PyMT parent, whereas introduction of the DBA/2J genome significantly reduced the density of pulmonary metastatic lesions (see Fig. 1; Ref. 20). These results suggested the presence of metastatic efficiency modifier alleles in the DBA/2J genome but not in the AKR/J genome. Therefore, the AKXD RI panel was used to identify candidate regions of the DBA/2J genome that suppressed metastatic efficiency. F1 progeny were generated from 18 of the AKXD sublines (average 9.6/line; range 4–16), the pulmonary metastatic density determined and linkage analysis performed. Linkage analysis of the cross revealed two loci at which the LOD scores were substantially above the empirically determined genomewide 5% threshold of significance (Fig. 2; Table 1). One of the key advantages of this RI backcross design is that the female F1 progeny are isogenic but recombinant. The genetic structure of the RI backcross progeny resembles a conventional N2 but with a much higher load of recombination breakpoints per genome. Therefore, the development and severity of tumor metastasis can be estimated accurately for each of the recombinant backcross progeny.
conventional backcross-mapping progeny were generated and analyzed. Previous studies demonstrated that F₁ hybrids between FVB/N-TgN(MMTV-PyMT)634Mul and DBA/2J or NZB/B1NJ suppressed metastatic involvement 10-fold compared with the FVB/NJ homozygous animals without altering tumor latency or tumor growth kinetics (20). Therefore, these strains are likely to contain modifier alleles that specifically affect the metastatic process. I/LnJ F₁ hybrids, in addition to suppressing metastatic density, also displayed altered tumor latency (38 days after birth versus 60 days; Ref. 21) and tumor burden (approximately 70–80%; Ref. 22) compared with FVB/NJ homozygotes. No evidence of a correlation between tumor latency with metastatic efficiency was observed (data not shown). A modest effect was observed for tumor growth rate (r = 0.49; Ref. 20), which reduces the power to detect metastasis specific modifier genes in these animals. However, this backcross was included in the analysis in the hope that there would be enough statistical power to detect one or more of the loci detected in the AKXD experiment at or above the recommended statistically suggestive threshold, thereby strengthening genomic localization data. The number of animals generated in each cross is indicated in Table 2.

Complete genome scans were performed for the backcrosses and chromosomal associations with suppression of metastatic efficiency determined as above (see Table 3 and Fig. 3). The NZB backcross demonstrated suggestive linkage to the same region of 19 seen in the AKXD experiment. Suggestive linkage was also observed on distal

Table 1 LOD scores for AKXD RI mapping experiment

<table>
<thead>
<tr>
<th>Locus</th>
<th>Chr 6</th>
<th>Chr 19</th>
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<tbody>
<tr>
<td></td>
<td>LOD (μ)</td>
<td>LOD (p)</td>
</tr>
<tr>
<td>Significance threshold</td>
<td>2.88</td>
<td>2.68</td>
</tr>
<tr>
<td>Mtv23</td>
<td>0.52</td>
<td>0.26</td>
</tr>
<tr>
<td>D6Mio33</td>
<td>3.31</td>
<td>0.32</td>
</tr>
<tr>
<td>Rs7a6</td>
<td>0.99</td>
<td>0.80</td>
</tr>
<tr>
<td>D6Nio3</td>
<td>0.22</td>
<td>0.74</td>
</tr>
<tr>
<td>Tgfa</td>
<td>0.00</td>
<td>0.12</td>
</tr>
<tr>
<td>D6Nio2</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>Rho</td>
<td>0.00</td>
<td>0.84</td>
</tr>
<tr>
<td>Raf1</td>
<td>0.36</td>
<td>1.20</td>
</tr>
<tr>
<td>D6Mio15</td>
<td>0.27</td>
<td>0.02</td>
</tr>
<tr>
<td>Xnum:54</td>
<td>0.05</td>
<td>0.00</td>
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</tbody>
</table>

* Genome-wide significance thresholds were determined by Permutation testing.

Table 2 Size of experimental crosses

<table>
<thead>
<tr>
<th>Mapping experiment</th>
<th>Animals analyzed</th>
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<tbody>
<tr>
<td>I/LnJ</td>
<td>125</td>
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<tr>
<td>DBA/2J</td>
<td>177</td>
</tr>
<tr>
<td>NZB/B1NJ</td>
<td>69</td>
</tr>
<tr>
<td>AKXD</td>
<td>171</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>544</strong></td>
</tr>
</tbody>
</table>

Table 3 LOD scores for backcross analysis

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>DBA/2J</th>
<th>I/LnJ</th>
<th>NZB/B1NJ</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>1.6</td>
<td>1.5</td>
<td>2.1</td>
<td>5.2</td>
</tr>
<tr>
<td>13</td>
<td>1.7</td>
<td>0.2</td>
<td>1.7</td>
<td>3.6</td>
</tr>
<tr>
<td>17</td>
<td>0.3</td>
<td>2.4</td>
<td>3.0</td>
<td>5.6</td>
</tr>
<tr>
<td>19</td>
<td>0.5</td>
<td>1.0</td>
<td>2.3</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* Significant threshold

* Genotype-wide significance thresholds were determined by Permutation testing.
Chr 17 and weaker linkage to Chr 13, as well as linkage to central Chr 9. The I/LnJ cross also exhibited suggestive linkage to distal Chr 17 as well as linkage to the proximal third of Chr 9. The DBA backcross also exhibited linkage for Chrs 9 and 13.

Surprisingly, in light of the AKXD experiment, no linkage was observed for suppression of metastatic efficiency and either the Chr 6 or Chr 19 loci in the DBA/2J backcross. Replication of the association between proximal Chr 19 in the NZB/B1NJ backcross strongly suggested the correct assignment of a metastatic efficiency modifier to this region. Previous studies in our and other laboratories (21, 39–41) have demonstrated the presence of unanticipated epistatic interactions in QTL analysis that might account for the discrepancy. Alternatively,
novel alleles may have become fixed in the inbred descendents of any of the inbred strains. Therefore, the genotype-phenotype correlation of the AKXD RI experiment was examined to determine which genome (AKR/J or DBA/2J) was associated with metastatic progression. Metastatic suppression at both loci was associated with the AKR/J rather than the DBA/2J alleles (see Fig. 4). Because the original strain survey demonstrated that (FVB/NJ × AKR/J)F1 animals were indistinguishable from FVB/NJ homozygous animals (20), the suppression attributable to these loci is most likely because of either epistatic interactions with DBA/2J loci or the presence of a locus in AKR/J that neutralizes the metastatic suppression in the (FVB/NJ × AKR/J)F1 hybrids that has been lost in the generation of the AKXD RI lines or recently gained in the AKR/J since the generation of the AKXD RI panel.

The coincident identification of the Chr 19 locus associated with metastatic suppression in the AKXD RI and NZB/B1NJ backcross experiments strongly suggest the presence of a metastasis modifier at metastatic suppression in the AKXD RI and NZB/B1NJ backcross panel. Recent gains in the AKXD RI experiment were examined to determine which genome of the inbred strains. Therefore, the genotype-phenotype correlation of novel alleles may have become fixed in the inbred descendents of any inbred strains. However, they were included in the analysis because it was anticipated that concordance between the backcrosses might potentially identify genomic regions specific to the metastatic process that might not be detected in any single experiment.

DISCUSSION

In this study we have described the generation and analysis of four different genetic mapping experiments (AKXD/Ty, DBA/2J, NZB/B1NJ, and I/LnJ; see Table 2) to identify the approximate genomic location of metastasis efficiency genes before initiating high-resolution mapping studies and positional cloning strategies to identify the genes of interest. Because of the complexity of the process, it was anticipated that a large number of genes might influence tumor dissemination and that metastasis-suppressing alleles of these genes might not be shared among inbred strains from relatively diverse backgrounds. To increase the probability of detecting significant associations as well as hopefully identifying multiple metastasis regulatory genes, multiple mapping experiments were analyzed. Three of the experiments involved inbred strains that altered only the metastatic phenotype of the mammary tumor (DBA/2J, NZB/B1NJ, and AKXD/Ty). The I/LnJ backcross altered tumor growth rate (22) and tumor latency (21), as well as metastatic efficiency. In the latter context the reduction in metastatic efficiency was expected to be the result of both reduction in tumor volume as well as specific genetic modification of tumor dissemination. The reduced metastatic efficiency because of decreased tumor volume would reduce the power of these experiments to detect loci specifically affecting tumor dissemination. However, they were included in the analysis because it was anticipated that concordance between the backcrosses might potentially identify genomic regions specific to the metastatic process that might not be detected in any single experiment.

Analysis of the results of these experiments reveals a number of points. First, not surprisingly, metastastic efficiency is a complex multigenic trait. No single modifier gene accounts for the significant variation in metastatic efficiency between inbred strains. Second, the reproducibility of the localizations suggests that there is likely to be only one or two pathways, at least in this tumor-metastasis model, that effect metastatic potential. If there were significantly more pathways involved it is unlikely that the loci would be replicated in multiple crosses. The polyoma middle-T antigen interacts and activates the PI3k/Akt signaling pathways and it has been demonstrated recently that constitutively active Akt can rescue the tumorigenicity of a
mutant polyoma middle-T protein that is incapable of interacting with PI3k. Interestingly, although tumorigenicity is restored in the Akt transgenic animals, metastatic capacity is not, suggesting that one or more pathways downstream of PI3k are required for tumor dissemination (45). It is likely that components of this pathway or pathways are the metastasis modifiers detected in this study. Third, the Mtes1 locus is likely to be specific for the metastatic process, not simply suppressing tumor dissemination by reducing tumor growth rates. Three of the mapping experiments were initiated using inbred strains that do not alter tumor growth kinetics (AKXD, NZB/BINJ, and DBA/2J); therefore, the loci detected are likely to be specific to the metastatic process. To confirm this, the data were reanalyzed with the tumor burden data to look for potential epistatic interactions that might affect growth kinetics. No significant associations were observed, additionally suggesting that the loci detected were specifically modifying tumor dissemination (data not shown). In addition, the I/LnJ backcross the metastasis-associated chromosomes detected in this study were independent of the chromosomes that harbor tumor growth modifier alleles (22). Identification and characterization of these genes will hopefully, therefore, provide valuable insights into the specific events and mechanisms required for tumor cells to disseminate and develop into secondary lesions. Chromosomal substitution or congenic strains are currently being constructed to confirm each of the metastatic efficiency suppressor intervals, explore potential genetic interactions, and initiate high resolution mapping studies.

On the basis of the analogy of inherited mutations in tumor suppressor genes predisposing individuals to cancer, an analysis of the metastasis suppressor genes was carried out to determine whether any of the known metastasis suppressors might be a candidate for Mtes1. The genomic locations of the known metastasis suppressor genes were identified in the Mouse Genome Informatics database to see if they colocalized with Mtes1. Comparative mapping and mining of the Radiation Hybrid maps demonstrated that the Brms1 locus colocalizes with the peak of the Mtes1 mapping data. Brms1 was identified as a gene commonly deleted in breast cancer metastases but not primary tumors and was shown to specifically suppress metastatic ability after transfection into highly metastatic cell lines. The colocalization of Brms1 and the mammary tumor metastasis efficiency gene Mtes1 raises the intriguing possibility that Mtes1 might be an allelic form of Brms1. Genomic analysis of this locus is currently in progress. However, preliminary evidence suggests that the differences in metastatic susceptibility observed in the various strains is not likely to be attributable to significant differences in transcription levels of Brms1 (data not shown).

The Chr 6 locus detected in the AKXD/Ty genetic mapping experiments was not replicated in the backcrosses, and, therefore, did not meet our stringent criteria applied to be assigned a locus designation. However, it is interesting to note that this region of the mouse genome contains homology to human Chr 7. In particular, the region between the loci Mtv23 and D6Mit33 (see Table 1), at the peak of the LOD score plots, is homologous to human 7q21–7q35, which, like the BRMS1 region, is often deleted in metastatic breast cancer (31). The Met oncogene, which has been implicated in tumor dissemination in a number of studies (46–48), is also present on mouse Chr 6, although it is proximal to Mtv23, and, therefore, unlikely to be a candidate for the potential metastasis efficiency modifier gene that might lie in this region. No other obvious candidate genes (metastasis suppressors, metalloproteinases, adhesion molecules, and so forth) reside in this region. Although the association of mouse equivalent of the human 7q metastasis-associated loss of heterozygosity region in this study is suggestive of the presence of a metastasis efficiency modifier, additional studies will be required to confirm the linkage studies by replication.

Although it is clearly too early to address the mechanism of the metastasis efficiency modifiers, the association of Mtes1 with the mouse homologue of BRMS1 raises an intriguing possibility. BRMS1 was recently shown to restore gap junction to human breast carcinoma cells (49). None of the loci described in this study colocalize with members of the connexin family of gap junction proteins. However, we describe previously the mapping of tumor growth modifier genes in the I/LnJ cross to mouse Chr 4. Chr 4 harbors four known gap junction genes (Gja3, 4, 5, and 10; Ref. 42), two of which are known to colocalize with the major growth modifier peak (22). We had demonstrated previously that the total tumor mass at time of sacrifice had a modest effect on metastatic potential (20) and had attributed part of the reduction of metastatic efficiency of the I/LnJ backcross to an indirect effect of reduced tumor mass. However, the colocalization of connexin gene family members with this peak suggests that a more direct effect may be in responsible.

Finally, this study suggests that there are subsets of human breast cancer patients that have significantly elevated risks of tumor dissemination. This inherited predisposition would help explain why some patients with relatively small tumors have extensive disseminated disease, whereas other patients with larger tumors have only localized lesions. Additional research into the genetic basis of the differences in metastatic efficiency will have two potential benefits. First, identifying and characterizing the genes responsible for the different metastatic efficiencies will likely provide greater understanding of the mechanisms responsible for tumor dissemination. This may permit the development of better antimetastatic therapies or preventative interventions. Second, by identifying the underlying genetic basis of efficient metastatic spread, it may be possible to identify that subset of breast cancer patients who are at highest risk. These patients could be examined more thoroughly to search disseminated tumors that may not yet be clinically apparent in order to treat them at the time of primary therapy. In addition, high-risk patients could be followed up more carefully and frequently after treatment for the primary tumor, resulting earlier detection and treatment of recurrent disease. Furthermore, it might be possible to enroll high-risk patients in chemoprevention protocols to inhibit or prevent the growth of clinically occult lesions, reducing the morbidity and mortality associated with metastatic breast cancer.

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MAMMARY TUMOR METASTATIC PROGRESSION AND BRMS1


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