

IDENTIFICATION OF INBRED MOUSE STRAINS HARBORING GENETIC MODIFIERS OF MAMMARY TUMOR AGE OF ONSET AND METASTATIC PROGRESSION

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Metastasis is one of the most important and complex processes in human neoplastic disease. A large number of both positive and negative events must occur to permit a tumor cell to colonize a distant site successfully. To identify mouse strains that harbor dominant genetic modifiers of this process, a strain survey was initiated utilizing a transgenic mouse mammary tumor model that exhibits a high incidence of pulmonary metastases. The transgenic animal was bred to 27 different inbred strains of mice and scored for the metastatic organ tropism and metastatic density. Thirteen strains were identified that had a statistically significant reduction in the numbers of pulmonary metastases. In addition, 10 strains were identified that altered the kinetics of induction of the primary mammary tumor. These strains will likely provide useful model systems for the analysis of genetic interactions in the initiation and progression of mammary adenocarcinomas. *Int. J. Cancer* 77:640–644, 1998.

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The mouse has been increasingly utilized as a model for the analysis of complex genetic human phenotypes (Frankel, 1995). The mouse models of human complex traits that have been successfully developed to date include ethanol addiction (Belknap *et al.*, 1993), diabetes (de Gouyon *et al.*, 1993), susceptibility to tumorigenesis (Ghosh *et al.*, 1993), kidney disease (Iakoubova *et al.*, 1995), neural tube defects (Neumann *et al.*, 1994) and obesity (Warden *et al.*, 1995). The availability of high-resolution mouse genetics and the large number of well-characterized inbred strains make the mouse a powerful system for studying multigenic or quantitative trait human disease phenotypes (Frankel, 1995).

A particularly important complex disease phenotype is that of tumor dissemination, or metastasis. Metastasis is one of the most important aspects of neoplastic disease and one of the most poorly understood (for review, see Liotta and Stetler-Stevenson, 1993). Although significant progress has been made in the elucidation of the metastatic process, a comprehensive understanding has been hampered by the complexity of the process and the intricacies of the tumor-host interaction (Liotta and Stetler-Stevenson, 1993). It has become apparent that development of metastatic disease is a multistep process that requires both positive and negative regulatory events to complete the metastatic cascade. Positive events include the activation of oncogenes and growth factors and induction of proteases, cellular adhesion proteins, motility factors and angiogenesis. Negative, or loss of function, events include inactivation of tumor suppressor genes and growth inhibitor processes, and the loss or inactivation of metastatic suppressors (Liotta and Stetler-Stevenson, 1993; Welch *et al.*, 1994).

Evidence for the genetic control or modulation of metastasis was generated from a number of studies, utilizing somatic cell hybrid fusions between non-metastatic and metastatic tumor cells (Miele *et al.*, 1996; Ramshaw *et al.*, 1983; Welch *et al.*, 1994). The resulting hybrids, while retaining their tumorigenic potential, were unable to metastasize. Subsequently, a prostate cancer metastatic suppressor locus was localized on human chromosome 11 in the region 11p11.2 and was eventually shown to be KAI1, a leukocyte surface glycoprotein (Dong *et al.*, 1995). Analysis of murine melanoma and human breast cancer cell lines have also revealed the specific down-regulation of the gene NM23, a nucleoside

5'-phosphate (NDP) kinase in metastatic tumors or cell lines vs. non-metastatic samples (Steege *et al.*, 1991).

Additional evidence for the genetic modulation of metastasis was obtained by a series of transfection experiments into murine cells (Tuck *et al.*, 1990). It was determined that a variety of activated proto-oncogenes, including H-RAS, v-mos, v-raf, A-RAF, v-src, v-fes, v-fms and p53, can induce tumorigenicity and metastatic potential when transfected into NIH-3T3 cells. However, when the same oncogenes were transfected into cell lines derived from different strains of mice, metastatic potential, but not tumorigenicity, was lost (Tuck *et al.*, 1990). This suggests that certain alleles present in some of the inbred strains of mice, either alone or in combination, can function as a metastasis suppressor. At present, these loci have yet to be characterized.

Although the mouse has been used as a model for a number of individual steps in the metastatic cascade, a mouse model for the entire process has not been developed. This is possibly a result of the fact that spontaneous metastatic mouse tumors have long latencies (Liebelt *et al.*, 1968), and the animals may succumb to the primary tumor or require sacrifice before the metastatic process can be completed. In addition, the poor penetrance of the metastatic phenotype of spontaneous mouse tumors (Liebelt *et al.*, 1968) would significantly complicate the analysis. A number of transgenic animals, however, have been found to metastasize extensively, possibly due to the accelerated nature of the disease (Anand *et al.*, 1994; Guy *et al.*, 1992; Nielson *et al.*, 1995; Wilkie *et al.*, 1994; Yang *et al.*, 1995). Since these animals develop metastatic disease in a heritable and highly penetrant manner, they offer the potential to utilize the power of mouse genetics to identify and characterize the modifier/suppressor loci known to be present in the mouse genome. One particularly interesting transgenic mouse is the MMTV-polyoma middle T (MMTV-PyMT) transgenic animal, which develops mammary tumors and extensive pulmonary metastases (Guy *et al.*, 1992). The MMTV-PyMT transgenic mouse develops synchronously appearing multifocal tumors involving all the mammary glands. Females develop palpable tumors within 60 days of birth, independent of pregnancy. Males also develop mammary tumors, although with a longer latency. In addition, more than 90% of the animals develop pulmonary metastases by 100 days (Guy *et al.*, 1992). The high penetrance and extensive metastatic potential of this animal make it an excellent model to perform genetic screens for metastasis modifier/suppressor genes.

To develop a model system for identification and characterization of dominant metastasis modifier/suppressor genes, our labora-

Grant sponsor: American Cancer Society; Grant number: IRG-191A; Grant sponsor: Department of Defense Breast Cancer Research Program; Grant number: DAMD17-7077; Grant sponsor: National Institutes of Health; Grant number: CA06927; Grant sponsors: MRC of Canada; CBCRI; Commonwealth of Pennsylvania.

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Received 30 January 1998; Revised 14 March 1998

tory has performed a genetic survey of more than 25 inbred strains. Virgin female transgene-positive F_1 progeny from (MMTV-PyMT \times inbred) outcrosses were generated and aged for the induction of the primary tumor and subsequent metastasis. The F_1 progeny were subsequently scored for metastatic target organ and density of pulmonary metastases. Thirteen inbred strains were identified that demonstrated a statistically significant reduction in the density of pulmonary metastases. Unexpectedly, 10 inbred strains were also identified that demonstrated significantly different latencies in the development of the primary mammary tumors. Utilization of these inbred strains and the MMTV-PyMT transgenic mouse in quantitative trait and standard genetic mapping experiments will likely provide novel genes for analysis of the complex process of mammary tumor formation and malignant progression.

MATERIAL AND METHODS

Mice

The FVB/N-TgN(MMTVPyMT) transgenic mouse utilized was the 634 line developed in the laboratory of W. Muller. I/LnJ, C58/J, ST/J, KK/HiJ, BUB/BnJ, NOD/LtJ, MOLF/Ei, SWR/J, AKR/J, CBA/CaJ, NZW/LacJ, DBA/1J, DBA/2J, 129/J, P/J, SEA/GnJ, A/J, LP/J, CE/J, RF/J, C57BL/10J, NZB/B1NJ, FVB/NJ and C57BR/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). CAST/Ei was a kind gift from Dr. M. Brilliant, Fox Chase Cancer Center (Philadelphia, PA). C57BL/6JNlcr, BALB/cAnNlcr and C3H/HeNlcr mice were purchased from the Laboratory Animal Facility of the Fox Chase Cancer Center. Tail biopsies were taken from weanlings to screen for germline transmission of the transgene. The MMTVPyMT was detected by PCR amplification with the following primers: 5'-AAC GGC GGA GCG AGG AAC TG-3': 5'-ATC GGG CTC AGC AAC ACA AG-3'.

Strain survey

FVB/N-TgN(MMTVPyMT) males were bred to females from each of the inbred strains. Female transgenic F_1 s were screened by palpation 3 times a week for the presence of the primary mammary tumor. The location of the tumor and the weight of the animal were recorded. The animals were examined for an additional week to confirm diagnosis and then aged for 40 days post-diagnosis to permit development of metastases. After 40 days, the animals were sacrificed by carbon dioxide inhalation; total carcass weight was determined, autopsies performed, and the lungs harvested for histological examination.

Histological analysis

Tissues were fixed in 10% paraformaldehyde, embedded in paraffin, serial sectioned and hematoxylin-eosin stained. For the determination of pulmonary metastatic density, 3 coronal non-adjacent sections of both lungs, each separated by 100 μ m, were prepared from each animal. The slides were examined with a Leica M420 Macroviewer with an Apozoom lens under 10 \times magnification with the objective 10 cm above the stage. Three fields were scored for each slide, for a total of 9 fields per animal. Pulmonary metastatic density was determined utilizing a Leica Q500MC Image Analysis System. All slides were read blind, and analysis was performed by a single operator to minimize operator bias. The macro used is available upon request.

Northern blot analysis

RNA was isolated from primary tumor tissue using RNeasy Maxi Kit (Qiagen, Santa Clara, CA), following the manufacturer's protocol; 15 μ g of total cellular RNA per sample were fractionated on 1% formaldehyde gels, stained with EtBr, and transferred to Hybond-N⁺ (Amersham, Arlington Heights, IL) by capillary transfer. Probes were labeled with the Prime-It kit (Stratagene, La Jolla, CA) and hybridized in Church's solution, as described by Church and Gilbert (1984).

RESULTS

Preliminary characterization of metastatic variables

To determine the variables that influence the metastatic phenotype, a preliminary characterization of the effect of length of tumor exposure, and of approximate tumor burden, on the density of pulmonary metastases was performed. Virgin FVB/N-TgN(MMTVPyMT) females were generated and monitored for the appearance of the primary tumor. At diagnosis of the primary tumor, each animal was weighed, and then aged for 20–70 days. Animals were then sacrificed, and total carcass weight was determined. A crude approximate of tumor burden was determined by subtracting weight at sacrifice from weight at diagnosis. No cachexia was observed in the MMTVPyMT animals; therefore, the change of weight should be a result predominantly, although not exclusively, of tumor tissue accumulation. Pulmonary metastatic density was determined from each animal and plotted as a function of either tumor period (defined as number of days between diagnosis and sacrifice) or tumor burden. In agreement with previous studies (Liebelt *et al.*, 1968), no significant correlation with tumor period and pulmonary metastatic density was observed (data not shown). In contrast to previous reports (Liebelt *et al.*, 1968), a weak correlation was observed ($r = 0.49$), however, between approximate tumor mass and pulmonary metastatic density (Fig. 1).

Strain survey

To identify inbred mouse strains that harbor dominant genetic modifiers of mammary gland carcinogenesis and progression, the MMTVPyMT animal was bred to 27 different inbred strains of mice. The inbred strains from different branches of the mouse phylogenetic tree and ferally derived strains were selected to maximize the probability of detecting strain-specific variations. Transgene-positive female F_1 s from each cross were monitored for the appearance of the primary tumor and then aged approximately 40 days to permit metastatic progression. The approximate tumor burden was calculated as described above. Three phenotypes were scored for each animal: tumor latency, metastatic target organ and density of metastases. No strain-specific variation in metastatic organ was observed. Significant variation was observed in the latency of the primary tumors (Table I) for a number of strains. Two strains, I/LnJ and C58/J, demonstrated statistically significant acceleration (20.6 and 17.8 days earlier, respectively; $p < 10^{-15}$ and 10^{-10} , respectively) of the primary tumor compared with the FVB/N-TgN(MMTVPyMT) animal. Seven additional strains demonstrated statistically significant delays of ≥ 7 days, compared with FVB/N, in the appearance of the primary tumor (C57BL/6JNlcr, NOD/LtJ, SWR/J, AKR/J, BUB/BnJ, ST/J, KK/HiJ and MOLF/Ei; $p < 0.01 - 10^{-6}$).

Histological examination of the lungs was performed utilizing a Leica Q500MC Image Analysis System to quantitate the number of metastases in each sample. The results were normalized to the amount of lung tissue scored to reduce errors due to variations in sample sizes on each slide. Since a weak correlation between the number of metastases and the amount of primary tumor tissue was observed (Fig. 1), the density of metastases (defined as the number of metastases per unit area of lung) was normalized to the approximate tumor burden. The resulting data, termed the metastatic index, was averaged for each inbred strain combination and compared with the FVB-TgN(MMTVPyMT) by Student's *t*-test to determine statistical significance (Table II). Thirteen inbred strains demonstrated statistically significant reduction of the density of pulmonary metastases compared with the FVB-TgN(MMTVPyMT) parent strain, with reductions ranging from 2- to 25-fold. Only one strain, AKR/J, demonstrated a consistent, although not statistically significant, increase in the density of pulmonary metastases, of approximately 3-fold.

Analysis of transgene expression

To determine whether the change in latency of the primary tumor or the change in pulmonary metastatic density could be correlated

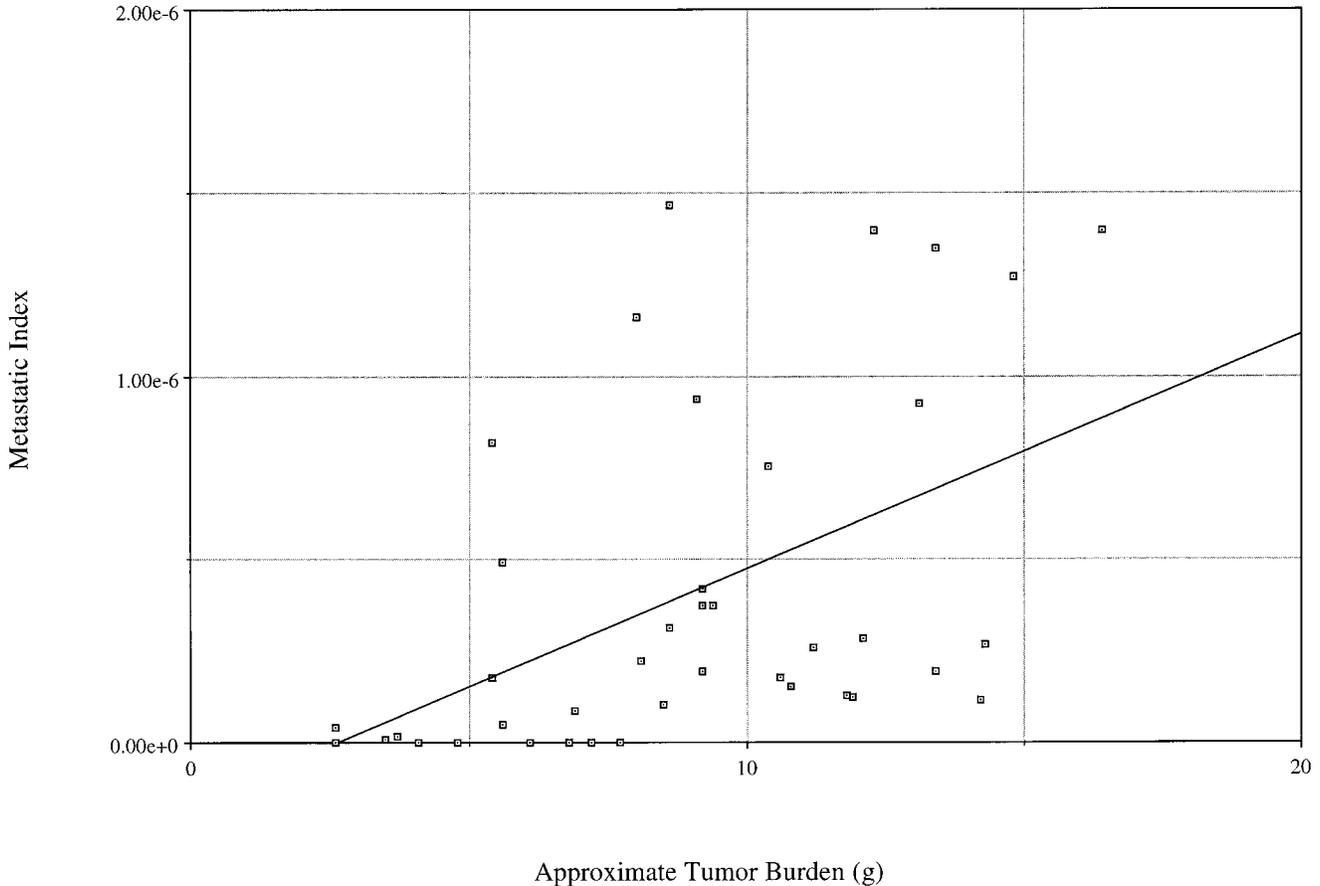


FIGURE 1 – Effect of approximate tumor mass, as measured by change in weight, on metastatic index. FVB/N transgenic females were aged for various periods after diagnosis of the primary tumor and sacrificed; approximate tumor burden and pulmonary metastatic densities were then determined. The data were entered into the program Cricket Graph v. 1.3.2 and analyzed as a scatterplot, and a best fit line was calculated.

with level of transgene expression, Northern blots were performed. Total cellular RNA from representative primary tumors from animals were fractionated on formaldehyde gels and hybridized with PyMT and β -actin probes. No correlation of increased transgene expression with decreased latency or reduction of metastatic index was observed (Fig. 2). In fact, a slight decrease in transgene expression was observed in the I/LnJ and C58/J F_1 tumors analyzed, suggesting that the decreased latency was due to additional modifier genes, rather than a direct effect of transgene expression.

DISCUSSION

Our study was designed to identify dominant genetic modifier alleles that modulate the ability of tumors to metastasize. Several variables that might influence the metastatic phenotype, including length of tumor exposure and approximate tumor mass, were analyzed in FVB/N-TgN(MMTVPyMT) animals to determine the appropriate endpoint and analytical methods. As expected, no significant correlation was observed between the length of time exposed to the tumor (defined as number of days between diagnosis of primary tumor and sacrifice) and the density of pulmonary metastases. This suggests that although the expression of the MMTVPyMT transgene is likely sufficient to induce the primary tumor, transgene expression does not provide all the additional signals or events required for metastatic progression. Since all these animals are presumably expressing the transgene with identical temporal kinetics, random secondary events must be

occurring to explain the wide variance observed in metastatic ability. Since tumor period did not significantly influence the density of metastases observed, 40 days after diagnosis of the primary tumor was selected for the experimental endpoint.

The second variable that might influence the measured metastatic ability was the approximate total tumor mass. A previous study had not demonstrated a correlation with the number of metastases and the amount of spontaneously arising primary tumors (Liebelt *et al.*, 1968). We performed preliminary experiments to determine whether there might be a correlation in this transgenic model. In contrast to the previous study, a weak correlation was observed. A possible explanation of the correlation would be that the greater the potential target tissue, the greater the probability of successfully completing the multiple steps required for metastatic progression. Therefore, all the determination of metastatic density index was normalized to approximate tumor mass to account for this weak correlation. The tumor mass utilized in this study is only an approximation. Due to the number of animals involved in our study, it was not feasible to dissect all the tumor tissue from each of the more than 400 animals studied. Since cachexia was not observed in the FVB/N parent or any of the outcross progeny, we believe that the change in weight of the animals between primary diagnosis and sacrifice is predominantly due to tumor tissue, with 2 notable exceptions. These are the F_1 progeny of the cross to I/LnJ and C58/J. I/LnJ and C58/J develop primary tumors prior to achieving adult size, and therefore the tumor burden as measured reflects both tumor tissue and normal

TABLE I – EFFECT OF MATERNAL GENOTYPE ON PRIMARY TUMOR LATENCY

Maternal genotype	Average latency (days)	SD	Median age (days)	Change in latency (days)	<i>p</i> value vs. FVB
I/LnJ	37.32	6.84	36	-20.60	2.17E-16
C58/J	40.11	6.82	39	-17.81	3.66E-11
LP/J	51.25	17.29	50	-6.67	0.45
129/J	53.58	12.43	51.5	-4.34	0.20
A/J	53.83	12.40	52	-4.09	0.39
BALB/cAnNIcr	56.44	9.96	58	-1.49	0.43
C3H/HeNIcr	57.80	13.10	58	-0.12	0.80
FVB/N	57.92	9.93	57	0.00	1.00
DBA/2J	59.44	12.03	59	1.51	0.84
NZB/B1NJ	59.64	7.86	61	1.72	0.68
C57BR/cdJ	59.88	11.42	62.5	1.95	0.73
C57BL/10J	60.31	11.54	59	2.38	0.66
CE/J	60.45	7.05	61	2.53	0.51
RF/J	61.20	8.23	64	3.28	0.56
SEA/GnJ	61.25	9.09	61.5	3.33	0.29
P/J	61.47	7.28	62	3.55	0.22
DBA/1J	64.00	11.59	62	6.08	0.19
NZW/LacJ	64.00	11.51	68	6.08	0.18
C57BL/6JNIcr	64.44	10.54	66	6.51	0.01
CAST/Ei	65.17	9.56	66	7.24	0.17
NOD/LtJ	66.65	9.53	69	8.73	3.95E-03
CBA/CaJ	66.67	13.08	68.5	8.74	0.07
SWR/J	66.74	14.46	67	8.82	9.71E-03
AKR/J	68.54	11.74	67	10.62	0.01
BUB/BnJ	69.64	7.64	68.5	11.72	1.64E-04
ST/J	74.00	8.79	77	16.08	2.69E-06
KK/HiJ	76.00	12.25	76	18.08	7.28E-05
MOLF/Ei	80.63	16.78	83.5	22.70	7.38E-03

TABLE II – EFFECT OF MATERNAL GENOTYPE ON PULMONARY METASTATIC DENSITY

Maternal genotype	Metastatic index	SD	No.	Metastatic index ¹ relative to FVB/N	<i>p</i> value vs. FVB/N
FVB/N	3.58E-08	4.60E-08	45	1.00	1.00E+00
RF/J	8.63E-10	1.93E-09	5	0.02	ND
C58/J	1.38E-09	2.19E-09	17	0.04	9.43E-06
C57BR/cdJ	2.35E-09	4.16E-09	16	0.07	1.52E-04
NZB/B1NJ	2.68E-09	3.82E-09	23	0.07	1.82E-05
I/LnJ	2.94E-09	6.15E-09	18	0.08	2.37E-05
DBA/2J	4.30E-09	6.64E-09	14	0.12	5.02E-05
KK/HiJ	6.09E-09	6.71E-09	13	0.17	1.19E-04
MOLF/Ei	7.08E-09	8.61E-09	5	0.20	ND
SEA/GnJ	8.39E-09	1.12E-08	23	0.23	2.82E-04
NZW/LacJ	1.24E-08	2.01E-08	10	0.35	0.02
CE/J	1.27E-08	1.42E-08	11	0.35	6.17E-03
ST/J	1.37E-08	2.01E-08	11	0.38	3.47E-04
C57BL/6JNIcr	1.47E-08	3.28E-08	34	0.41	0.02
P/J	1.58E-08	1.14E-08	15	0.44	9.64E-03
DBA/1J	1.66E-08	4.09E-08	10	0.46	0.21
NOD/LtJ	1.68E-08	1.40E-08	19	0.47	0.02
A/J	2.00E-08	2.16E-08	6	0.56	0.18
C3H/HeNIcr	2.02E-08	2.48E-08	20	0.56	0.08
SWR/J	2.46E-08	3.93E-08	21	0.69	0.30
CBA/CaJ	2.94E-08	2.36E-08	12	0.82	0.51
BUB/BnJ	4.35E-08	3.39E-08	14	1.22	0.50
129/J	4.47E-08	3.21E-08	12	1.25	0.13
BALB/cAnNIcr	4.51E-08	2.72E-08	18	1.26	0.33
C57BL/10J	6.71E-08	8.58E-08	13	1.87	0.23
CAST/Ei	6.75E-08	9.61E-08	5	1.89	0.51
AKR/J	1.09E-07	1.12E-07	13	3.04	0.07
LP/J	0.00E+00	0.00E+00	3		ND

¹Metastatic index, number of metastases/lung area (μm²)/tumor burden.

growth. Complete dissection of the tumors from a number of these animals demonstrates that the metastatic index would only be altered by 2-fold, still resulting in a highly significant reduction in the density of pulmonary metastases.

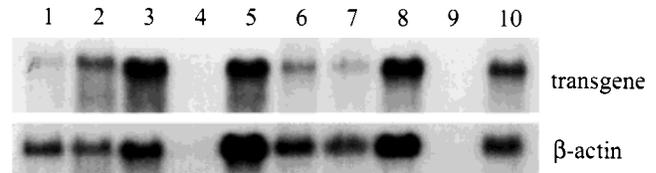


FIGURE 2 – Northern blot analysis of representative primary tumor samples. Lane 1: SEA/GnJ; lane 2: C57BL/6JNIcr; lane 3: NZB/B1NJ; lane 4: DBA/2J; lane 5: C57BR/J; lane 6: I/LnJ; lane 7: C58/J; lane 8: ST/J; lane 9: MOLF/Ei; lane 10: FVB/N. The DBA/2J and MOLF/Ei samples are degraded.

Thirteen different inbred strains were identified that had a statistically significant reduction in the metastatic index. The strains derive from many different branches of the mouse phylogenetic tree, suggesting the existence of multiple modifier genes. Additional evidence for the presence of multiple metastasis modifier genes or alleles can be inferred from the variance of the phenotype, ranging from between 2.5- to 25-fold reduction in average metastatic index compared with FVB/N. The broad range in reduction of metastatic index is most likely explained by multiple genes that were segregated into the various strains during the genesis of inbred mice. One strain, AKR/J, demonstrated a consistent approx. 3-fold increase in the metastatic index. This result is not statistically significant with the number of animals analyzed to date, but it is trending toward statistical significance. The AKR/J outcross is currently being repeated to generate enough animals to determine whether this result is significant. If significant, this inbred strain would be very useful for the discovery and analysis of dominant metastatic enhancers. Three additional strains, LP/J, RF/J and MOLF/Ei, also demonstrated a tendency toward a statistically significant reduction of metastases. However, since the number of animals scored was small, *p* values were not calculated. Outcrosses with the MOLF/Ei are being continued to evaluate the significance of the result. The LP/J and RF/J lines have been discontinued due to breeding difficulties.

Unexpectedly, in addition to dominant effects on the metastatic index, variations in the age of onset were also observed for a number of strains. The F₁ progeny of 2 outcrosses (I/LnJ and C58/J) had significantly shorter latency periods than the FVB/N parent. Eight additional strains had an increased latency, of approximately a week or greater. The most likely explanation for this variation would be alterations in the transgene expression, as was observed comparing other transgenic lines (Guy *et al.*, 1992). Northern blot analysis of the tumors from the F₁ progeny analyzed so far, however, does not demonstrate significant differences in levels of expression. Another possibility would be that the different genetic backgrounds are affecting the temporal expression of the transgene. *In situ* hybridization and immunohistochemical strategies are currently being developed in our laboratory to address this question.

The metastasis modifier genes detected in our study are likely to be generally applicable, not specific to the PyMT transgene model. In contrast to what has previously been proposed, our data suggest that expression of the PyMT transgene does not provide all the signals required for metastatic progression (Ritland *et al.*, 1997). The wide variance in metastatic phenotype between genetically identical animals and the correlation of tumor mass with metastatic density suggest that additional events must occur to permit successful tumor dissemination. These events are likely to be specific to the multiple events required to implement the metastatic pathways successfully, rather than the tumor induction mechanism and are therefore more likely to represent truly genetic interactions in human neoplastic disease. Ultimately, however, similar types of

genetic analyses will have to be performed with metastatic modifier genes as with latency modifying genes, to confirm involvement in human metastatic progression. Nonetheless, the inbred strains identified in our genetic strain survey will likely provide an extremely useful model system for the identification and characterization of the genetic modification and interactions in at least some aspects of mammary tumor neoplasia.

ACKNOWLEDGEMENTS

The authors thank Drs. M. Brilliant, W. Kruger and E. Henske for useful discussions and Dr. E. Moss for manuscript review. WM was the recipient of grants from the MRC of Canada (Research Scientist) and CBCRI; an appropriation from the Commonwealth of Pennsylvania was given to KWH.

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