Loss of Heterozygosity Analysis in Primary Mammary Tumors and Lung Metastases of MMTV-MTAg and MMTV-neu Transgenic Mice

Steve R. Ritland, Gerald J. Rowe, Yue Chang, and Sandra J. Gendler

Department of Biochemistry and Molecular Biology, Mayo Clinic Arizona, Scottsdale, Arizona 85259

ABSTRACT

Loss of heterozygosity (LOH) analysis has been used in many types of human cancer to localize putative tumor suppressor genes important in carcinogenesis. However, this approach has only recently been applied to transgenic mouse tumor models, which offer greater opportunity for detailed molecular genetic analysis of tumor initiation and progression. To explore the possible role of secondary genetic events in transgenic mouse mammary tumor development, we performed microsatellite-based allelotyping on primary mammary adenocarcinomas and lung metastases arising in mice transgenic for the polyomavirus middle T antigen under the control of the mouse mammary tumor virus promoter/enhancer (MMTV-MTAg mice). We examined a total of 80 microsatellite loci distributed throughout the mouse genome for LOH and observed high rates of specific chromosomal loss but very low rates of background allelic loss in these tumors. For the MMTV-MTAg mice, no individual chromosomes showed rates of LOH significantly above the background rates. For MMTV-neu mice, markers on chromosome 4 showed LOH in 82% of mammary tumors, whereas markers on chromosome 3 showed loss in 29% of tumors. These data suggest that the middle T antigen transgenic mice do not undergo whole chromosome loss or large genomic deletions as common mechanisms of tumor formation and that chromosomes 3 and 4 may contain tumor suppressor gene loci that play important roles in the development of neu-mediated mouse mammary tumors.

INTRODUCTION

Oncogene activation and TSG inactivation are fundamental genetic mechanisms of tumor development. There has been much success in recent years in identifying and cloning the genes involved in the pathogenesis of specific tumor types (reviewed in Ref. 1). LOH is one of the techniques that has been used to describe the genetic loci likely to contain TSGs, and LOH has aided in the localization and positional cloning of these genes. Although most LOH studies to date have been done using human tumor systems, interpretation of these studies has been complicated by the high degree of genetic instability frequently associated with human cancers. Transgenic animal tumor models provide a useful alternative system to study cancer genetics because they offer several important advantages including defined genetic background, controlled environment, and a lower overall rate of genetic instability.

Mice transgenic for oncogenes under the control of tissue-specific promoters facilitate a detailed analysis of the molecular mechanisms involved in oncogene-mediated tumor development. These transgenic models have been used to successfully study the in vivo signal transduction pathways of individual oncogenes (2) and to investigate the cooperative interactions of multiple oncogenes (3, 4). Because transgenic animal models provide a stable, renewable resource for analyzing tumors at a specific developmental stage, they provide a unique opportunity to study the secondary genetic events of oncogene-mediated tumor development. The development of a high resolution simple sequence length polymorphism map of the mouse genome has made detailed genetic deletion mapping possible through LOH analysis in interspecific hybrid transgenic mouse tumor models. Although several studies using LOH analysis with chemically induced mouse tumors have been published recently (5–11), LOH studies using transgenic animal tumor models have only recently begun to appear (12–14).

The transgenic mammary tumor models used in this study have been described in detail elsewhere (15, 16). Briefly, mice expressing the polyomavirus middle T antigen under the control of the MMTV promoter/enhancer (MMTV-MTAg mice) undergo rapid production of multifocal mammary adenocarcinoma with a high rate of secondary metastasis to lung. The mechanism of MTAg-mediated mammary epithelial cell transformation is thought to involve the association of MTAg with members of the c-Src family of receptor tyrosine kinases and its interaction with phosphatidylinositol 3'-kinase (17–20). The short latency period, extremely rapid tumor kinetics, and synchronous occurrence of multifocal mammary tumors in MMTV-MTAg mice suggest that secondary genetic alterations may not be required for primary tumor formation in this system (15).

The second transgenic model used in this study involves mice expressing the neu (c-erbB2) proto-oncogene under the control of the MMTV promoter/enhancer (MMTV-neu mice). The neu proto-oncogene encodes a Mr 185,000 transmembrane tyrosine kinase protein that is a member of the epidermal growth factor receptor family (21). Overexpression of neu has been implicated in the development of human breast cancer and has been identified as a negative prognostic indicator (22–24). The MMTV-neu transgenic mice used in this study overexpress unactivated neu in the mammary epithelium and develop focal, frequently metastatic mammary adenocarcinoma after a relatively long latency period (16). The mechanism of neu-mediated mammary epithelial cell transformation is thought to involve an activating somatic mutation in the portion of the neu gene coding for the extracellular region of the transmembrane protein (25). Activated neu is thought to exert its mitogenic effect on mammary epithelial cells through direct interaction with c-Src, resulting in elevated c-Src tyrosine kinase activity (2, 26).

The role of secondary genetic events in two these transgenic mammary tumor models is not adequately defined. In the case of MMTV-MTAg mice, the rapid development of multifocal tumors suggests that additional genetic lesions may not be required for primary tumor formation, i.e., polyomavirus middle T antigen alone may be sufficient to fully transform mouse mammary epithelial cells in vivo and to confer metastatic potential (27). Jakubczak et al. (28) examined mutation rates in mice bitransgenic for the polyomavirus middle T antigen and a bacteriophage lambda reporter gene and concluded that a significant increase in mutagenesis was not required for mammary tumor development in the MTAg system. However, it is important to note that not all tumors in MMTV-MTAg mice progress in a uniform and synchronous manner, and not all mice with established primary...
mammary tumors show gross evidence of metastatic disease.3 Therefore, it is possible that secondary genetic events or other epigenetic factors modulate the expression of the tumor phenotype in MMTV-MTAg mice. In the case of MMTV-neu mice, the long latency period and frequently unifocal occurrence of tumors suggest the requirement of one or more additional genetic lesions downstream of unactivated neu overexpression for tumor formation. Although the oncogenic activation of neu through somatic mutation may alone account for the observed latency period and phenotypic variation (29), we hypothesized that other genetic deletion events may also play a role in this neu-mediated mammary tumor system.

This study examined genome-wide LOH in two transgenic mouse mammary tumor models, MMTV-MTAg and MMTV-neu. In the MMTV-MTAg system, we have assayed a total of 42 polymorphic microsatellite markers in 47 primary mammary adenocarcinomas and 22 secondary lung metastases. In the MMTV-neu system, we have examined 76 microsatellite loci in 22 primary mammary adenocarcinomas. We observed very low rates of background allelic loss in both of these tumor systems, and we found no evidence of microsatellite instability. However, we observed a high frequency LOH on chromosomes 3 and 4 in primary mammary tumors from MMTV-neu mice, implying that these chromosomes may contain TSGs that play a role in the development of neu-mediated mammary tumors.

MATERIALS AND METHODS

Mice. The MMTV-MTAg (line 634) and MMTV-neu (line 202) transgenic mice were obtained through a kind gift from Dr. W. J. Muller (McMaster University, Hamilton, Ontario, Canada) and were maintained in a specific pathogen-free facility. Mice were screened for germ-line transmission of the transgenes using the following PCR methods; MTAg PCR amplified a 491-bp region of the polyomavirus middle T antigen gene. The primers used were: forward primer, 5'-AGT CAT TGA ATT CAC ACC GAG-3' and reverse primer, 5'-AGT AGC CCC AGC CAC ACC-3'. PCR conditions were 94°C for 3 min and then 40 cycles of 94°C for 30 s, 61°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min. neu PCR amplified a 560-bp region of the MMTV 3' long terminal repeat promoter region of the MMTV-neu transgene. The primers used were: forward primer, 5'-CAG GTG CAA GGA CTA TTA TGA C-3' and reverse primer, 5'-CTG ACA AGG CAC ATC AGA ACC-3'. PCR conditions were 94°C for 5 min and then 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min. The MMTV-MTAg mice used in these LOH studies were generated through two serial matings of FVB-MIAg transgenic male founders with (B6 × 129)12 outbred females. The MMTV-neu mice were generated by mating FVB-neu males with B6 females to generate (B6 × FVB-neu)F1 animals. There was no significant difference in tumor incidence or multiplicity between F3, B6, and FVB × (B6 × 129)12, B6, and FVB. Primer pairs were synthesized on an ABI 394 DNA synthesizer (Applied Biosystems, Foster City, CA) or were purchased from Research Genetics (Huntsville, AL). A list of the microsatellite markers that were used in this study is available upon request, and all primer sequences are available through the Whitehead M.I.T database.

PCR and Electrophoresis. Purified genomic DNA (25–50 ng) was amplified in a PCR reaction mix containing 1× PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2), 0.2% formamide, 200 µM (each) of dATP, dGTP, and dTTP, 40 µM dCTP, 0.4 µM of each primer, 0.03 unit/µl Taq DNA polymerase (Boehringer Mannheim), and 0.06 µCi/µl [α-32P]dCTP (3000 Ci/mmol; Amersham). The PCR reaction volume was 25 µl/tube. Thermal cycling was performed in Perkin-Elmer 9600 instruments using the following parameters: an initial incubation at 95°C for 5 min followed by 26–30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min. Following PCR amplification, each reaction was mixed with 1 volume of loading buffer (95% formamide, 10 mM EDTA, 1 mg/ml bromophenol blue, and 1 mg/ml xylene cyanol green) and heat denatured for 5 min at 95°C. 3 µl of each mixture was electrophoresed through denaturing polyacrylamide gels (% polyacrylamide, 7 M urea, 15% formamide, and 0.5× TBE (0.045 M Tris-borate, 0.001 M EDTA, pH 8.0)) at 75 W for 1–2 h. Gels were vacuum dried and exposed to Kodak BioMax MR film for 12–48 h.

Assessing LOH. Autoradiographs were interpreted visually by comparing allelic intensities of normal versus tumor DNA at polymorphic loci. A reduction in signal intensity of >50% in one of the tumor alleles was interpreted as LOH. To confirm these findings, many of the critical PCR reactions were repeated and were analyzed using a Packard (Meridan, CT) Instant Imager, which provided quantitative verification of LOH. The strain of origin for the affected allele was determined based on our initial polymorphism screens using pure-strain DNA templates. The allelic loss maps were assembled using linkage data from the Mouse Genome Database.

Statistics. Statistical comparisons between the frequency of individual chromosome LOH with the background rate of LOH were made using a two-tailed Fisher’s exact test. Comparison of strain preference for allelic loss on chromosome 4 was made using a one-sample binomial test using 50% as the null binominal probability.

RESULTS

Microsatellite Markers. A large panel of microsatellite markers were initially screened for polymorphism between the 129, B6, and FVB inbred strains, and 80 markers were selected for use based on

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3 Unpublished observations.
polymorphism, linkage position, and ease of interpretation. Because the transgenic models used in this study had different genetic backgrounds, not all 80 markers were used on all tumors. However, all tumors were assayed using at least one informative marker per autoimmune.

**LOH Analysis.** Fig. 1 shows example autoradiograph images demonstrating LOH for markers on chromosomes 3 and 4 in MMTV-neu mammary tumors. A reduction in allelic intensity of >50% was classified as LOH, and visual comparison was verified by repeated PCR and quantitative measurement using a Packard Instant Imager instrument. No significant bias for deletion of the heavier allele was observed in any of the tumors analyzed.

**MMTV-MTAg Analysis.** Twenty-three microsatellite markers were used to assess allelic loss in 47 primary mammary adenocarcinomas obtained from virgin female MMTV-MTAg mice 4 months of age. The overall rate of LOH observed (number of tumors showing LOH for one or more locus on a chromosome/total number of tumors informative for that chromosome) in these tumors was 2.0%, and no single chromosome showed rates of loss significantly above background. Fig. 2A shows the frequency distribution of LOH by chromosome for MMTV-MTAg mammary tumors.

We also assayed 52 microsatellite markers in 22 secondary lung metastases from MMTV-MTAg mice. The overall rate of LOH observed in these tumors was 2.7%, and no single chromosome showed rates of loss significantly above background. Fig. 2B shows the frequency distribution of LOH by chromosome for MMTV-MTAg lung metastases.

**MMTV-neu analysis.** Seventy-six microsatellite markers were used to assess allelic loss in 22 spontaneous mammary adenocarcinomas from MMTV-neu mice 7–14 months of age. The overall rate of LOH observed in these tumors was 10.4%, and chromosomes 3 and 4 showed rates of loss significantly above background ($P = 0.027$ and $P < 0.001$, respectively). Fig. 2C shows the frequency distribution of LOH by chromosome for MMTV-neu mammary tumors.

**Allelic Imbalance on Chromosomes 3 and 4 in MMTV-neu.** The rate of allelic loss observed in MMTV-neu mammary tumors was 82% (14 of 17 tumors) on chromosome 4 and 29% (5 of 17 tumors) on chromosome 3. The pattern of loss for these chromosomes is illustrated in Fig. 3. Whole chromosome loss appears to be the most frequent pattern observed on chromosome 4, with 12 of the 14 animals showing LOH for all informative markers. Only two tumors showed partial loss of chromosome 4, with tumor 5 retaining heterozygosity for the proximal marker D4Mit172 (13.6 cm) and tumor 13 showing no loss at D4Mit148 (66.0 cm) and D4Mit64 (71.4 cm). These recombinant intervals were confirmed by duplicate PCR reactions and by densitometry.

**Strain Bias for LOH on Chromosome 4.** Preferential allelic loss with regard to strain of origin was observed for loci on chromosome 4 (Fig. 3). For tumors showing LOH on chromosome 4, 86% (12 of 14) lost either the B6 or 129 allele, whereas 14% (2 of 14) lost the FVB allele. Based on the breeding scheme used to generate these animals, all polymorphisms involved one copy of the FVB allele; therefore, the expected frequency of FVB allele loss was 50%. The observed rate of FVB allele loss is significantly less than the expected rate ($P = 0.0018$).

**DISCUSSION**

The MMTV-MTAg and MMTV-neu mouse tumor models provide powerful systems in which to study the genetic pathways of mammary tumor development. To explore the phenotypic consequence of loss-of-function mutations occurring downstream of oncogene overexpression in the mouse mammary gland, we performed whole-genome LOH analysis in primary mammary tumors and their associated pulmonary metastases.

**LOH in MMTV-neu Mouse Mammary Tumors.** The highly specific incidence of LOH observed on chromosomes 3 and 4 in MMTV-neu tumors strongly suggests that these chromosomes harbor genes that play an important role in neu-mediated mammary tumor development in the mouse. Although mitotic nondysjunction giving rise to whole chromosome loss appeared to be the most common mechanism of deletion observed, tumors 5 and 13 showed evidence of partial chromosome 4 loss, possibly due to chromosomal recombination, terminal deletion (tumor 5), or interstitial deletion (tumor 13). The observed strain bias for allele loss in these tumors (B6 or 129 in preference to FVB) may indicate the existence of one or more mammary tumor susceptibility loci on chromosome 4 in the FVB genome, or it may reflect the influence of genomic imprinting.

Several previous studies examining LOH in chemically induced mouse tumors have also shown chromosome 4 to be a frequent target of deletion (7–8, 11). This suggests that mouse chromosome 4 may contain one or more TSGs that play important roles in multiple tumor...

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* List available upon request.
Fig. 2. Allelotypes of MMTV-MTAg and MMTV-neu transgenic mouse mammary adenocarcinomas and lung metastases. A, frequency of LOH in 47 MMTV-MTAg primary mammary tumors using 23 polymorphic microsatellite loci. The overall rate of LOH in these tumors was 2.0%. B, frequency of LOH in 22 MMTV-MTAg secondary lung metastases using 32 polymorphic microsatellite loci. The overall rate of LOH in these tumors was 2.7%. C, frequency of LOH in 22 MMTV-neu primary mammary tumors using 76 polymorphic microsatellite loci. The overall rate of LOH in these tumors was 10.4%, and rates of loss on chromosomes 3 (29%) and 4 (82%) were statistically significant (*; \( P = 0.027 \) and \( P < 0.001 \), respectively).
types. Mouse chromosome 4 shares synteny with human chromosomes 1p, 6q, 8q, and 9, with the most extensive synteny to regions on 1p and 9. Allelic loss studies in human sporadic breast cancer have revealed frequent LOH on chromosomes 1p and 9 (reviewed in Ref. 30). In the case of chromosome 1p, two or more distinct regions of loss have been identified, although no definitive TSGs have yet been cloned from this chromosomal segment (31—33). In the case of chromosome 9, the cyclin-dependent kinase inhibitor CDKN2/p16INK4a/MTS1 residing on chromosome 9p21 has recently been implicated as a TSG that may play a role in human breast cancer development (34, 35). p16INK4a and its alternate transcript p19ARF have also been shown to undergo deletion and mutation in several other types of human (36) and murine (37, 38) cancer and are known to play a regulatory role in progression through the cell cycle (39). The related protein p15INK4b has also been suggested to have tumor suppressor activity (40, 41). The genes encoding p16INK4a and p15INK4b have both been mapped to cytogenetic position C3—C6 on mouse chromosome 4 (41).

Another candidate tumor susceptibility locus on mouse chromosome 4 is Mom-1, which is known to modulate the expression of the intestinal tumor phenotype in ApcMin mice (42). Phospholipase A2 (Pla2a2/Pla2g2a), which lies on mouse chromosome 4 at 66.6 cM, has been identified as a candidate gene for Mom-1 (43). In human breast cancer, the expression level of membrane-associated phospholipase A2 (M-PLA2, mapping to human chromosome 1p35) has been shown to be an independent prognostic indicator (44, 45).

Because of the small number of losses observed in this data set, further studies using a higher marker density and more tumors will be necessary to identify common regions of deletion and possible candidate genes on chromosome 3.

**LOH in MMTV-MTAg Mouse Mammary Tumors and Secondary Lung Metastases.** MMTV-MTAg and MMTV-neu mice share a common viral promoter element in their transgene constructs (15, 16). The virtual absence of LOH in MMTV-MTAg system is also of interest because the high incidence of allelic imbalance observed on chromosomes 3 and 4 in MMTV-neu tumors reflects the inactivation of TSGs rather than strain-specific promoter modulatory elements. The absence of LOH in the MMTV-MTAg system is also of interest in the secondary lung metastases because not all MMTV-MTAg mice appear to undergo synchronous metastasis (see description of tumors in "Materials and Methods"). These data suggest that in vivo overexpression of the middle T antigen may alone be sufficient to drive mammary tumorigenesis through metastasis.

In conclusion, the high incidence of LOH observed for chromosomes 3 and 4 in tumors from MMTV-neu mice strongly suggests that one or more tumor susceptibility genes on these chromosomes are involved in neu-mediated mouse mammary tumor development. Further mutation detection studies using the MMTV-neu model will reveal whether several candidate TSGs in these regions of LOH are involved in neu-mediated mouse mammary tumor development. The virtual absence of LOH in MMTV-MTAg mice suggests that the high incidence of allelic imbalance observed on chromosomes 3 and 4 in MMTV-neu tumors reflects the inactivation of TSGs rather than strain-specific promoter modulatory elements.

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