Elevated Frequency of Loss of Heterozygosity in Mammary Tumors Arising in Mouse Mammary Tumor Virus/neu Transgenic Mice

Marc Cool and Paul Jolicoeur

Laboratory of Molecular Biology, Clinical Research Institute of Montreal, Montreal, Quebec, H3W 1R7 Canada [M. C., P. J.]; Department of Microbiology and Immunology, University of Montreal, Montreal, Quebec, H3C 3J7 Canada [P. J.]; and Division of Experimental Medicine, McGill University, Montreal, Quebec, H3G 1A4 Canada [P. J.]

ABSTRACT

Loss of heterozygosity (LOH) analysis was performed on 62 mammary tumors that were induced in (BALB/c × C57BL/6)F1 mouse mammary tumor virus/neu transgenic mice. Eighty-six simple sequence length polymorphism markers were used to cover all of the somatic chromosomes. Frequency of LOH was observed to be significant for chromosomes 4 (50%), 19 (32%), and 8 (21%). On chromosome 4, at least three distinct regions of allelic deletions could be identified: one proximal to 22 cM; the second close to the p16INK4a/p15INK4b locus, which is commonly deleted in various tumors; and the third one in the proximity of Mom1. The frequency of LOH on chromosome 19 was the same for the four markers used. Our data suggested the presence of two distinct LOH loci, one proximal to 47 cM and the other at the distal region. On chromosome 8, possibly two distinct LOH loci could be recognized, one around 52 cM and the other one at 67 cM or distal to it. These regions map close to E-cadherin (Cdh1) and M-cadherin (Cdh15) loci, respectively. Because LOH sites are thought to harbor tumor suppressor genes, this allelotype analysis has allowed the mapping of putative tumor suppressor genes. The regions of allelic loss are likely to harbor a tumor suppressor gene.

INTRODUCTION

The erbB-2/neu oncogene codes growth factor receptor transmembrane tyrosine kinase (1, 2). This gene is frequently amplified and overexpressed in human breast cancers, and this change is thought to contribute at least partially to malignancy (reviewed in Ref. 3). Other genetic modifications, such as activation of other oncogenes or inactivation of tumor suppressor genes, in addition to the increased expression of the erbB-2/neu gene, are likely to be involved in the development of these malignant tumors, as it has been elegantly demonstrated in other types of human tumors (4). In fact, LOH of the most common type of mutation in human primary breast carcinoma (5). It affects at least 20 regions of the genome, encompassing the following human chromosome arms: 1p, 1q, 3p, 6q, 7q, 8p, 11p, 11q, 13q, 16q, 17p, 17q, 18q, 22q (6→8), 2p (9), 7p (10), 8q, 9p, 15q (11), 10q (9), 16p (12), Xq, and Yq (13). Some and maybe all of these regions of allelic loss are likely to harbor a tumor suppressor gene.

This concept has now been validated by the cloning of several tumor suppressor genes in regions of LOH (14). This idea stems from the two-hit model of genetic inactivation of tumor suppressor genes by Knudson (15), stating that inactivation of a tumor suppressor gene requires mutations affecting both alleles independently, the first event being a small germ-line mutation of a single allele, in the case of familial cancer, and the second being a mutational event on the other allele, often consisting of a large chromosomal deletion scored as LOH. The number and identity of most tumor suppressor genes involved in human breast cancers are unknown, and only a few of them have been identified; p53 (16), RB1 (17, 18), PTEN/MMAC1 (19, 20), E-cadherin (21, 22), FHIT (23, 24), TSG101 (25), and p16INK4a (26, 27) have all been found to be inactivated at various frequencies in sporadic breast carcinoma. In familial breast cancer, inactivation of BRCA1 and BRCA2 is a relatively frequent event (28, 29).

To study the molecular nature of the erbB-2/neu transformation event of the mammary glands, we (30) and others (31) have generated Tg mouse animal models (MMTV/neu), which are more amenable to experimentation. These mice harbor a transgene consisting of a full-length neu cDNA, with a Val-664→Glu mutation located in the transmembrane domain, expressed under the regulatory sequences of the MMTV promoter (30). These MMTV/neu Tg mice develop mammary carcinoma that was pathologically very similar to the human tumors. Although, mammary tumors have been reported to arise after a short latency period in some MMTV/neu Tg mice (31), most of these MMTV/neu Tg mice develop tumors stochastically and after a long latency (30, 32), strongly suggesting that the erbB-2/neu oncogene is not by itself sufficient for tumor formation and that other genetic events (such as activation of other oncogenes and inactivation of recessive oncogenes) are likely to contribute to the development of these malignant tumors.

In an attempt to identify novel tumor suppressor genes that may be involved, in collaboration with the activated erbB-2/neu oncogene, in the appearance of these tumors, we used the LOH analysis on tumors from (BALB/c × C57BL/6)F1, MMTV/neu Tg mice. For this study, we used 62 mouse mammary tumors from MMTV/neu Tg mice and performed a genome-wide analysis with 86 microsatellite SSLP markers to search for LOH sites. A few specific regions of LOH on 3 chromosomes were identified. We are proposing that these regions with allelic losses harbor tumor suppressor genes involved in the development of these mammary tumors, in collaboration with the erbB-2/neu oncogene.

MATERIALS AND METHODS

Mice. The MMTV/neu Tg mice line MN-10 has been described previously (30). They were initially produced in a (C57BL/6 × C3H)F1 background and were subsequently maintained on a BALB/c background by crossing with male or female BALB/c mice (Charles River, St-Constant, Quebec, Canada) for 10 generations. At the 10th generation, the MMTV/neu Tg males were bred with C57BL/6 females (Charles River, St-Constant) to obtain (BALB/c × C57BL/6)F1, MMTV/neu Tg female mice, which developed spontaneous mammary tumors. The frequency and characterization of these tumors have been described (30).

DNA Preparation. Mammary tumors and kidneys were collected from Tg females. Kidneys were used as source for normal DNA. DNA was extracted as described previously (33) in 10 mM Tris (pH 7.5), 0.4 mM NaCl, 2 mM EDTA, 0.5% SDS, and 0.5 mg/ml Pronase buffer for overnight at 65°C, followed by phenol extraction, then chloroform/isooamyl alcohol (24:1) extraction. DNA was precipitated in 2 volumes of ethanol.

Microsatellites. The 86 microsatellite markers (SSLPs) used (Table 1) have been described by Dietrich et al. (34, 35). They encompass all 20 somatic chromosomes and exhibit polymorphism at the C57BL/6 and BALB/c alleles.
The linkage position for these markers was found in the Whitehead MIT database. These DNA marker oligonucleotides were purchased from Research Genetics (Hunstville, AL).

**PCR.** Analysis for LOH was done by PCR amplification using one radio-labeled primer, the “forward” primer. The labeling mix reaction consisted of 0.05 μM “forward” primer, 1 μC/μl (3000 Ci/mmol) [γ-32P]ATP (Amer- sham), and 0.125 unit/μl T4 DNA polynucleotide kinase (Phar- macia) and was incubated for 30 min at 37°C, then heated at 100°C for 30 s. The PCR mix reaction was made in a 10-μl volume containing 20 ng of genomic DNA, 0.05 μM of labeled “forward” primer as well as 0.05 μM of unlabeled “reverse” primer, 200 μM of each of four deoxynucleotides (dATP, dCTP, dGTP, and dTTP), 0.125 unit/μl Taq polymerase (Pharmacia), and 1× PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1.5–2.5 mM MgCl2]. Amplification was performed in a Perkin-Elmer 9600 instrument with the following parameters: 94°C for 3 min, then 25 cycles of 94°C for 15 s, 55°–60°C for 2 min, 72°C for 2 min, and finally 72°C for 7 min.

**Analysis of SSLPs.** Loading buffer (2×; 0.08% bromphenol blue, 0.08% Xylene cyanol, and 10% glycerol) was added to amplified PCR mix reaction. Samples (5 μl) were loaded on a 6% nondenaturing PAGE and run in 1× TBE buffer (0.09 M Tris, 0.09 M boric acid, 2 mM EDTA). Each sample was always compared with its normal DNA (kidney) extracted from the same animal. The assessment of allelic loss (LOH) was visually scored on an autoradiographic film. Only tumors showing 50% or more decrease in intensity of one of the allelic fragments was scored as positive for LOH. More than two assays were done to confirm LOH. In case of a moderate decrease (25%) in intensity for an allelic fragment, it was scored as an AI. Statistical analysis to determine the significance of the percentage values of tumors with LOH for one chromosome against its normal DNA (kidney) extracted from the same animal. The overall rate of LOH was calculated as the number of tumors with LOH over the number of all informative tumors. Noninformative tumors retained some C57BL/6 genomic regions present in the (C3H × C57BL/6)F2 embryo inoculated initially with the transgene. This may reflect the fact that these Tg mice have not yet been bred on a BALB/c background for 20 generations. In addition, in one region of chromosome 7, whose a large portion has remained heterozygote, the transgene may have integrated in the C57BL/6 allele and been selected for. Strain bias was observed to deviate from the expected 50% distribution and was confirmed by a χ² statistical test.

### RESULTS

**Characteristics of the Mammary Tumors.** The MMTV/neu Tg mice (line MN-10) described previously (30) and used in the present study have been bred on a BALB/c background for 10 generations. Both male and female MMTV/neu Tg mice were crossed with normal C57BL/6 mice to produce F1 mice. The F1 Tg female mice were bred continuously to multiply the number of pregnancies and were allowed to age up to the point they spontaneously develop mammary tumors between the ages of 10 and 26 months. Sixty-two independent mammary tumors (1–3 cm in diameter) were collected from these mice. Some mice developed more than one tumor, and these were all collected and treated as independent tumors.

**LOH Analysis.** The DNAs from these 62 MMTV/neu Tg mammary tumors were analyzed by using 86 microsatellite markers (SSLPs) distributed on each chromosome. These markers were chosen according to their polymorphism, and an effort was made to have them evenly dispersed (≈20 cM; Table 1). Assuming that each marker covers 10 cM on each of its sides, we estimated that 76% of all 20 chromosomes were covered by our 86 markers. LOH was a very frequent event in these neu-induced tumors; as much as 56 of 62 tumors (90%) had at least one chromosome with LOH. The mean number of chromosomes having an LOH per tumor was 2.1, and the median frequency was 2.4. We found that the highest number of chromosomes with LOH in individual tumors could go up to 9 (Fig. 1).

The frequency of allelic loss on each chromosome is given in Fig. 2. The overall rate of LOH was 11%. On most chromosomes, the rate of LOH was low, around 5% or below (chromosomes 2, 3, 5, 6, 10, 11, 12, 13, 15, 17, and 18) or close to the average rate of 11% (chromosomes 7, 9, 14, 16, and X). Chromosome 1 showed LOH at a frequency of ~20%, but further analysis showed that this did not appear to be significantly different from the overall rate (see below). Interestingly, a much higher frequency of LOH was detected on chromosome 8 (21%), chromosome 4 (50%), and chromosome 19 (32%). The frequency of LOH was significantly different from the overall rate of 11% for chromosomes 4 and 19 (P < 0.001) and for chromosome 8 (P = 0.05).

To have a better picture of the specific location of the LOH on each chromosome, the frequency of LOH detected with each marker was plotted (Fig. 3). The average mean frequency of the LOH background...
of all chromosomes was 8.7%. With most of the markers, the frequency of LOH was 5% or below. A few markers scored LOH in ~10% of the tumors, not significantly different than the overall rate. Markers on chromosome 4 remained significantly different and could detect LOH in 28–48% of the tumors. Similarly, LOH on chromosome 19 was measured with four different markers in 24–26% of the tumors. The frequency of LOH on chromosome 8 was lower. One marker (D8Mit280) could detect LOH in only 3.2% of the tumors, whereas another one (D8Mit91) could detect LOH in 21% of the tumors (three times the value of the background). Comparison of the percentage of tumors with LOH detected with all four of chromosome 8 markers used showed a significant difference (14%) from the background mean of 8.7% (P = 0.042).

Together these results indicate that LOH on chromosomes 4, 19, and 8 is a frequent event in mammary tumors arising in MMTV/neu Tg mice.

**LOH on Chromosome 4.** Of 62 tumors, 43 (69%) showed LOH or AI on chromosome 4 (Fig. 4A). Nine, and possibly 12, tumors had complete deletions of a region spanning from 12.1 to 77.5 cM, which is almost the entire chromosome 4. This type of deletion accounts for 28% of the 43 tumors, and for 40% when considering the 31 tumors with LOH only. The highest percentage of LOH (48%) was found with the D4Mit54 marker (66 cM). Another peak of LOH (42%) was observed with the D4Mit178 marker (35.5 cM). Interestingly, despite the fact that several tumors have lost apparently the entire chromosome 4, a small number of tumors showed selective LOH + AI at each of the individual markers, except D4Mit127, independently of each other (Fig. 4B). Although the number of tumors exhibiting these characteristics is small, this observation suggests the presence of as many as four and possibly five independent distinct loci affected by allelic deletion along the chromosome 4. These regions have been designated Naad1 to Naad5 (Fig. 4B).

Our results also show a strong strain bias on allelic loss on this chromosome 4, because deletions of these alleles significantly deviate from an expected 50% distribution between BALB/c and C57BL/6 alleles. Of 31 tumors with LOH, only 7 had lost the C57BL/6 allele, whereas 24 tumors lost the BALB/c allele. This represents a significant difference (P < 0.002). No such strain bias on all other chromosomes, except chromosome 19 (below), was found in this set of tumors.

**LOH on chromosome 19.** Our analysis of chromosome 19 showed that the rate of LOH varies from 24 to 26%, along the chromosome from 26 to 54 cM (Fig. 5A). When the number of tumors with LOH + AI are added, this enables us to distinguish from the relatively high rate of allelic loss for the marker D19Mit19 (44%), relative to the three other markers at 39% (D19Mit10), 32% (D19Mit34), and 30% (D19Mit71). Moreover, these data suggest that possibly two distinct loci may be affected by allelic deletion, one (Naad6) at or proximal to D19Mit10 and the other (Naad7) at D19Mit34 or distal to it (Fig. 5B). The fact that a high proportion of tumors exhibiting allelic loss (LOH + AI) on this chromosome have lost most of the chromosome also suggests the presence of more than one allelic deletion site (Fig. 5A). Additional tumors will need to be analyzed to assess the number and correct percentage of LOH sites on this chromosome.

As with chromosome 4, a strain bias for allelic loss was observed. Fifteen tumors of 20 exhibited deletion of the C57BL/6 allele, whereas only five tumors lost the BALB/c allele (P = 0.025). However, this strain bias on allelic loss of chromosome 19 (C57BL/6 allele loss) was different from the one observed on chromosome 4 (BALB/c allele loss).

**LOH on Chromosome 8.** The highest percentage of tumors with LOH on this chromosome is 21% (Fig. 6A). The deleted region includes the D8Mit86 and D8Mit91 markers and spans from 52 to 67 cM between the D8Mit280 marker (72 cM) and the D8Mit69 marker (31 cM). Possibly two distinct loci may be affected by allelic deletion on this chromosome: one (Naad8) proximal to D4Mit91 and the other one (Naad9) distal to D8Mit86. The analysis of additional tumors may...
confirm the presence of these independent loci. No strain bias was observed for this chromosome.

DISCUSSION

The identification of additional genetic events, which might cooperate with the activated neu/erbB-2 oncogene to generate mammary carcinoma, appears to be important to understand the molecular basis of this neoplasia. To this end, we looked for recessive oncogenes that may be selectively lost in these tumors. To map such putative tumor suppressor genes, we used the allelic deletion or LOH approach on 62 mammary carcinoma DNAs from (BALB/c × C57BL/6)F1 MMTV/neu Tg mice with 86 microsatellite markers dispersed on all chromosomes. LOH was detected at a low frequency at several loci across the genome, with an overall rate of 8.7%. Although rare, these genetic events may not be insignificant for a given tumor but remain difficult to study experimentally. Interestingly, we also found that a few loci had sustained allelic deletion in a significant percentage (21–48%) of these tumors, indicating that these genetic events tended to be specific and frequent in this set of tumors. The markers showing the highest score for LOH were D4Mit54 (48%), D4Mit178 (42%), D4Mit236 (28%), D4Mit111 (32%), D4Mit12 (37%), D4Mit127 (35%) on chromosome 4 and D19Mit10 (26%), D19Mit34 (26%), D19Mit71 (26%) on chromosome 19. Another marker, D8Mit91, on chromosome 8 had a lower score (21%).

Our analysis revealed that possibly up to two independent putative tumor suppressor loci are present on chromosome 8. One of them (Naad8) mapped close to the E-cadherin (CDH1) gene (53.3 cM). It is syntenic to human 16q22.1 where the E-cadherin (CDH1) gene has been mapped. In human breast cancers, LOH has been observed in this 16q22.1 region (36), and E-cadherin has been found to be mutated as a tumor suppressor gene (22). The second (Naad9) locus mapped distal to 67 cM and colocalized with the aprt (adenine phosphoribosyl transferase) and Cdh15 loci. The human syntenic 16q24.3, where M-cadherin (CDH15) has been mapped, was reported to be deleted in human breast cancers (36, 37). Therefore, E- and M-cadherin (CDH1 and CDH15) remain candidate tumor suppressor genes on the mouse chromosome 8.

The LOHs detected on chromosome 19 cover a large region of the chromosome, with a possibility that two distinct loci are affected by LOH. One of these regions, Naad6, may represent the Psa3 LOH region proximal to D19Mit19 identified in other murine tumors, i.e., liver and lung tumors (38–40). However, deletion of chromosome 19 regions is an uncommon event in other mouse mammary tumors induced by other methods in different mouse strains (41–44). The human 11q region is syntenic to the proximal region of mouse chromosome 19 and is known to show LOH in breast carcinomas (45). The other human syntenic region of this chromosome, 10q23–25, harbors a known tumor suppressor gene, PTEN/MMAC1, deleted in a variety of human tumors, including breast cancers (19, 20). The allelic deletion locus (Naad6) observed for the D19Mit19 (26 cM) marker is in the vicinity of the mouse PTEN gene, which maps at 24.5 cM. The other putatively independent distal LOH region, Naad7, is syntenic to the human 10q25–26 region, which has not been reported to show LOH in human breast cancers. However, LOH of this region has been
observed in other types of human cancers, and candidate tumor suppressor genes mutated on both alleles have been identified: MXI1 in prostate cancer (46), DMBT1 in malignant brain tumor cell lines (47), and h-neu (human homologue of Drosophila neuralized gene) in malignant astrocytomas (48).

More than two and possibly five distinct loci (Naad1 to Naad5) exhibiting allelic deletion at relatively high frequency were found on chromosome 4. This suggests that the simultaneous loss of several of these putative tumor suppressor genes may favor tumor growth, and this could explain the loss of the entire chromosome 4 observed in a significant proportion of the neu-induced mammary tumors (15%). A very similar pattern of LOH has been reported to occur in thymic lymphomas (49, 50), in ras-induced mammary carcinomas (41), and in lung tumors (51). These investigators have postulated the presence of at least two and up to three distinct loci of LOH. The Naad1 and Naad2 loci may correspond to the proximal LOH locus reported by Radany et al. (41). This Naad1 region is in synteny with the human regions 8q11–q22, 6q14–21, and 9p13–q32, and the Naad2 region is syntenic to human 9p13-q34. The 6q14–15 region has been reported to exhibit LOH in breast cancer (52). LOH has been reported in the 8q22 region in adult acute myeloid leukemia (53). This region harbors no known candidate tumor suppressor genes. The Naad2 region is syntenic to the human 9q32–34, which harbors a candidate tumor suppressor gene known as DBCCR1, the promoter of which was found hypermethylated in bladder cancer (54). The Naad3 locus covers a region harboring two well-known genes, MTSl(p15INK4b) and MTSp2(p16INK4a), which have been reported to be deleted in mouse hepatocellular carcinoma cell lines (55–57), in thymic lymphomas (49, 50, 58), and in lung tumors (44, 59–61). In the latter tumors, homozygous deletions of the genes p15INK4b and p16INK4a have been observed (62). Although we have not observed homozygous deletions of the D4Mit178 marker in the neu-induced mammary tumors studied here, p15INK4b and p16INK4a remain candidate tumor suppressor genes in these neu-induced mammary carcinomas. Naad3 corresponds to the TLSR1 locus of Santos et al. (49). This Naad3 locus is syntenic with human 9p21–23, which harbors the p15INK4b and p16INK4a (CDKN2a and CDKN2b) genes, in addition to being syntenic with the 1p31–35 region. The former region is frequently deleted in different types of human cancers (63), including breast cancers (26, 64). However, inactivation of p16/CDKN2a is a rare event in primary breast tumors and was found predominantly in immortal breast epithelial cell lines (26). Moreover, it has been reported recently that p16INK4a (CDKN2a) expression was increased in human breast carcinoma (65). In addition, Radany et al. (41) found that these two p15INK4b and p16INK4a genes were unlikely to be involved in ras-induced mammary carcinomas. Together, these results suggest that these two genes may not be implicated in neu-induced mammary tumors, and that a novel tumor suppressor gene may be inactivated.
ACKNOWLEDGMENTS

We thank Michel Ste-Marie, Benoît Laganère, and Patrick Couture for excellent technical assistance. We are grateful to Alain Guimond and Stéphane Sirois for initial work on this project.

REFERENCES


Elevated Frequency of Loss of Heterozygosity in Mammary Tumors Arising in Mouse Mammary Tumor Virus/ neu Transgenic Mice

Marc Cool and Paul Jolicoeur


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/10/2438

Cited articles  This article cites 75 articles, 25 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/10/2438.full.html#ref-list-1

Citing articles  This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/59/10/2438.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.