Ethynitrosourea-induced Development of Malignant Schwannomas in the Rat: Two Distinct Loci on Chromosome 10 Involved in Tumor Susceptibility and Oncogenesis

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ABSTRACT

Inbred rodent strains with differing sensitivity to experimental tumor induction provide model systems for the detection of genes that either are responsible for cancer predisposition or modify the process of carcinogenesis. Rats of the inbred BD strains differ in their susceptibility to the induction of neural tumors by N-ethyl-N-nitrosourea (EtNU). Newborn BDIX rats that are exposed to EtNU (80 µg/g body weight; injected s.c.) develop malignant schwannomas predominantly of the trigeminal nerves with an incidence >85%, whereas BDIV rats are entirely resistant. A T:A→A:T transversion mutation at nucleotide 2012 of the neu (erbB-2) gene on chromosome 10, presumably the initial event in EtNU-induced schwannoma development, is later followed by loss of the wild-type neu allele. Genetic crosses between BDIX and BDIV rats served: (a) to investigate the inheritance of susceptibility; (b) to obtain animals informative for the mapping of losses of heterozygosity (LOH) in tumors with polymorphic simple sequence length polymorphisms (SSLPs); and (c) to localize genes associated with schwannoma susceptibility by linkage analysis with SSLPs. Schwannoma development was strongly suppressed in F1 animals (20% incidence). All of the F1 schwannomas displayed LOH on chromosome 10, with a consensus region on the telomeric tip encompassing D10Rat6, D10Mgh16 and D10Rat2 but excluding neu. A strong bias toward losing the BDIV alleles suggests the involvement of a BDIX-specific tumor suppressor gene(s). Targeted linkage analysis with chromosome 10 SSLPs in F2 intercross and backcross animals localized schwannoma susceptibility to a region around D10Wox23, 30 cM centromeric to the tip. Ninety-four % of F1 tumors exhibited additional LOH at this region. Two distinct loci on chromosome 10 may thus be connected with susceptibility to the induction and development of schwannomas in rats exposed to EtNU.

INTRODUCTION

Cancer development is not merely the result of random genetic alterations but also depends on individual genetic predisposition. Up to 5% of all human cancers are considered due to hereditary cancer predisposition syndromes (1, 2). Susceptibility genes—mostly heterozygous mutant tumor suppressor genes transmitted through the germline—account for the elevated cancer risk of individuals. Familial cancer syndromes are identified relatively easily because of highly penetrant genes. However, individuals suffering from seemingly sporadic tumors may also be genetically predisposed. This predisposition can be effected by functionally polymorphic alleles of genes that increase the risk for certain types of cancer, albeit with a probability much lower than 100% (1). Analysis of the polymorphic expression of cancer susceptibility/resistance genes is nearly impossible in humans because of the low penetrance of the genes with no obvious familial clustering, which precludes the identification of carriers (3).

Inbred rodent strains susceptible to carcinogenesis carry the alleles of predisposing gene(s) in a homozygous state. Homozygosity for mutant tumor suppressor genes is in most cases incompatible with embryonic development (3). Susceptibility genes of such strains are thus unlikely to belong to this category. Inbred rodent strains may, therefore, permit the identification of predisposing gene loci with low penetrance by linkage analysis in appropriate genetic crosses with resistant animals. This approach has been used in previous studies of susceptibility to a variety of chemically induced cancers, such as pulmonary adenomas (4), colon cancer (5, 6), mammary cancer (7), and skin cancer (8).

The identification of chromosome-specific LOH in tumor cells—allele-specific or random—represents another way to detect loci with a tumor suppressor function, either polymorphic and involved in inherited cancer predisposition or without functional differences between individuals. Here again, rodent genetic models are advantageous because large numbers of genetically identical, fully informative F1 offspring can be bred for tumor induction and subsequent allelic deletion mapping, with the parental origin of each allele being known (9). Using a combination of both strategies, we have attempted to map susceptibility loci for the development of chemically induced rat tumors of the PNS.

Inbred strains of BD rats (10) exhibit differential sensitivity to the induction of neural tumors by pulse-exposure to the alkylating carcinogen EtNU (11, 12). Although rats of the BDIX strain develop malignant schwannomas of the PNS, predominantly of the trigeminal nerve, with an incidence >85% after exposure to EtNU on postnatal day 1, rats of the BDIV strain are entirely resistant. A point mutation in the neu (erbB-2) gene localized on chromosome 10q32.1 (13) appears to be the initial event in the development of these tumors and later on is followed by the loss of the wild-type neu allele (14, 15). The mechanism of loss and the functional consequences of this genetic event in schwannoma development have remained unclear in so far as the mutant neu gene is considered a dominantly acting oncogene (see, e.g., Ref. 16). Theoretically, the neu LOH may be due to a second point mutation or, more likely, to a deletion or mitotic recombination event.

We have performed allelic deletion mapping on chromosome 10 in EtNU-induced schwannomas of BDIX rats and BDIV rats. LOH of different size were found, with the smallest region of overlap excluding neu and a strong strain-specific bias in favor of the BDIV alleles. Assuming that a putative susceptibility gene(s) on chromosome 10 could have tumor suppressor activity (as is the case for most cloned genes predisposing to cancer) and might thus be subjected to allelic deletion in rat schwannomas, a
whole genome scan was not carried out at this stage. Instead, targeted linkage analysis (17, 18) was performed on chromosome 10, using (BDIX × BDIV) $F_2$ intercross and (BDIX × BDIV) $F_1$ × BDIX backcross animals.

**MATERIALS AND METHODS**

**Rat Strains and Genetic Crosses**

Animals were bred and maintained at the animal facility of the Institute of Cell Biology (Cancer Research), Essen, Germany. Rats of the inbred strains BDIX (sensitive to schwannoma induction by EtNU) and BDIV (resistant to schwannoma induction by EtNU); Refs. 10, 11) served as founders of the reciprocal first generation offspring (BDIX × BDIV) $F_2$ and (BDIX × BDIX) $F_2$. $F_1$, $F_2$ animals of both orientations were then mated to produce reciprocal $F_2$ intercross progeny. Backcross animals were generated by crossing (BDIX × BDIX) $F_2$ rats with animals of the sensitive BDIX strain in both orientations. Genomic DNA was isolated from a piece of tail removed after weaning.

**Induction of Malignant Schwannomas by EtNU**

BDIV, BDIX, $F_1$, and $F_2$ intercross and backcross rats received a single s.c. injection of EtNU (80 μg/g body weight) 24 h postnatally (14, 15). Beginning at 10 weeks after exposure to EtNU, the rats were checked for neurological symptoms twice weekly. Animals exhibiting visible tumors, cachexia, shortness of breath, paralyses, or behavioral abnormalities were killed by CO$_2$. Complete gross necropsy examination was performed on each animal. Tumors were removed, snap-frozen in liquid N$_2$, and stored at −80°.

**Genotyping and Allelic Deletion Mapping by SSLP- and RFLP-PCR**

Genomic DNA was isolated from normal and schwannoma tissues using the “Quia Amp Tissue Kit” (Qiagen) and stored frozen in H$_2$O at 4 mg/μL. For genotyping of $F_2$ intercross and backcross progeny and for detection of allelic imbalances in tumors of $F_1$ and $F_2$ intercross animals, 64 SSLPs on chromosome 10 were initially screened with DNA isolated from rats of the parental strains BDIX and BDIV. Thirty-six SSLPs were polymorphic. Of these, the most diagnostic markers were used for linkage mapping and/or detection of allelic imbalances. Primers for microsatellites D10Rat96, D10Mgh25, D10Mgh12, D10Mgh11, D10Mgh10, D10Mih4, D10Mih3, D10Mih2, D10Wox15, D10Wox14, D10Wox6, D10Wox5, D10Wox19, D10Mih12, D10Wox7, D10Mgh2, D10Rat4, D10Rat3, D10Mgh16, and D10Rat21 (19, 20) were purchased from Research Genetics (Huntsville, AL). Primers for SSLPs within the ngfr and δδ genes (21) and for D10Wox23 (20) were obtained from Life Technologies (Eggersheim, Germany).

PCR reactions were carried out in a 10-μL volume with 20 ng of DNA in flexible 96-well plates (Advanced Biotechnologies LTD, Epsom, United Kingdom) using a Hybird Omn E Thermal Cycler (AGS, Heidelberg, Germany). The final concentrations of primers and dNTPs were 330 nm and 150 μM, respectively. 0.25 units of Taq polymerase (AmpliTaq; Perkin-Elmer/Cetus, Foster City, CA) were used per reaction. PCR conditions were those of the thermocycling protocol described in Ref. 19. PCR reaction mixtures were supplemented with 6 μL of loading buffer consisting of xylene cyanole and bromphenol blue (0.05% each) in 100% formamide with 20 mM EDTA, denatured at 90°C for 3 min, and electrophoresed on a 6% polyacrylamide denaturing gel (0.4 mm) in Tris-borate EDTA buffer at 85 W. Gels were silver-stained using a staining frame mounted to the larger glass plate (22).

**RESULTS**

**Inheritance of Susceptibility to Schwannoma Development in Crosses of BDIV and BDIX Rats.** To confirm the differential susceptibility toward schwannoma development previously observed for BDIV versus BDIX rats transplacentally exposed to EtNU (27), BDIX ($n = 21$) and BDIV rats ($n = 24$) were exposed to EtNU on postnatal day 1, i.e., the developmental window ensuring a maximum yield of trigeminal schwannomas (~90%) relative to schwannomas in other locations and to central nervous system tumors (14). Five BDIV animals were accidentally killed without detectable tumors or other disease. However, 14 (88%) of the remaining 16 BDIX rats developed trigeminal schwannomas after a median latency time of $t = 187$ days. Two animals were lost because of the early occurrence of other tumors. In contrast, none of the BDIV rats developed schwannomas of the trigeminal nerve nor at any other location in the PNS. Two rats were prematurely killed. Nineteen animals succumbed to malignancies of the central nervous system and three animals developed tumours at other locations after $t = 323$ days. BDIV rats exposed to EtNU thus proved to be resistant to schwannoma development (see Fig. 1A for survival data).

To determine the susceptibility of heterozygous animals to EtNU-induced schwannoma development and to perform LOH analysis of the resulting tumors, BDIV and BDIX rats were mated in both orientations. Fifty-five $F_1$ progeny were generated [33 (BDIX × BDIX) $F_2$ and 22 (BDIV × BDIV) $F_2$]. Schwannoma development was strongly suppressed in EtNU-exposed $F_2$ animals. Only 21.5% of the $F_2$ animals exhibited trigeminal schwannomas at autopsy with no significant differences in incidence and latency time between both orientations (average median latency period, $t = 298$ days). The majority of $F_2$ rats had to be killed because of the development of neoplasms of the central nervous system as well as of the kidney (censoring events for the analysis of survival times; see Fig. 1B).

For linkage mapping, segregating crosses (268 $F_2$ intercross and 141 backcross progeny) were exposed to EtNU. We found that 29.6%...
GENETIC SUSCEPTIBILITY TO RAT SCHWANNOMA INDUCTION

Fig. 1. Induction of malignant schwannomas by pulse-exposure to EtNU on postnatal day 1. Distribution of survival times for the parental inbred rat strains BDIX and BDIV and for their F1 and F2 generations. A, distribution of disease-free survival times for 21 BDIX rats (thick line) and 24 BDIV rats (thin line). Fourteen BDIX rats developed malignant schwannomas, two rats exhibited other types of tumors, and five rats (marked with stars) were killed without disease when neurological symptoms were wrongly assumed. BDIV rats did not develop schwannomas but showed tumors of the central nervous system. B, Kaplan-Meier estimate of the distribution of times until the occurrence of symptoms caused by schwannomas for 54 (BDIX × BDIV) F2 rats (thick line) and 268 (BDIX × BDIV) F2 rats (thin line) of both orientations. Animals with symptoms caused by tumors other than schwannomas or other diseases were counted as “censored observations”; their survival times are marked with ticks.

Fig. 2. A, LOH on chromosome 10 in EtNU-induced rat schwannomas. The polymorphic markers used are listed on the left. Centromeric to telomeric from top to bottom. ●, loss of the BDIV allele; □, loss of the BDIX allele; shaded bars, maximum possible deleted region for each tumor; □□□, retention of heterozygosity; no symbol, not informative. The chromosomal positions of the markers are shown on the right. B, genetic linkage map of rat chromosome 10 based on genotyping data from 268 animals of both sexes of a (BDIX × BDIV) F2 intercross, as determined with the use of 24 polymorphic markers (listed on the right). Distances (cM; on the left) were calculated using the mapmaker program Mapmaker 3.0 with the Kosambi function, a linkage map spanning 105 cM of chromosome 10 was constructed with an average distance of 4.53 cM between markers (Fig. 2B). In the region covering the smallest region of overlap for the LOH and the susceptibility locus (in “Results”), no markers were separated by more than 6.0 cM.

LOH Analyses of Chromosome 10 in Trigeminal Schwannomas (F1 and F2 Generation). Seventeen trigeminal schwannomas (∼3 mm in diameter) induced in F1 rats and exhibiting the T:A→A:T transversion mutation at nucleotide 2012 of the neu gene, diagnostic of EtNU-induced rat schwannomas (14), were initially analyzed for LOH of chromosome 10 with the use of 19 polymorphic markers. All (17 of 17) of these tumors showed LOH of chromosome 10, with a common region of deletion at the last three subtelomeric markers (D10Rat3, D10Mgh16, and D10Rat2; Fig. 2. A and B), thus excluding the neu gene. This locus was termed Dis1 for deleted in schwannomas (Fig. 2B). The chromosome-10 LOH of most (16 of 17) of the schwannomas, however, encompassed the neu gene as well as two or more marker loci centromeric to neu. Fourteen of these tumors retained the allele originating from BDIX. Twenty-six of the tumors generated in F2 rats were informative for different portions of chromosome 10, and four tumors were homozygous for all of the markers tested (data not shown). LOH for all of the informative markers occurred in 10 tumors; 9 tumors displayed allelic losses for informative markers in the more distal portion of the chromosome exclusively, retaining heterozygosity in the centromeric region. Seven tumors exhibited no loss. Of the latter, two schwannomas (No. 173 and No. 26) retained heterozygosity in the common region of LOH (Dis1) as defined in the F1 tumors. Twelve of 19 schwannomas generated in F2 rats retained the allele originating from BDIX. The results of the LOH analyses are summarized in Fig. 2A.

Linkage Analyses. Because F1 schwannomas predominantly lost genetic material of BDIV origin on chromosome 10—pointing to the existence of a strain-specific negative regulatory element (tumor sup-
Table 1  Association of the susceptibility to the induction of malignant schwannomas by EtNU with genetic markers on chromosome 10

Data represent the genotypes of schwannoma-bearing animals of the F2-intercross and backcross generation. The association with chromosome 10 SSLPs was calculated using the TDT.

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<th>TDT statistic value</th>
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* ND, not determined.

**DISCUSSION**

We have used a combination of LOH analysis and targeted linkage mapping to localize functionally polymorphic genes with tumor suppressor activity involved in genetic predisposition to the development of EtNU-induced malignant schwannomas. Loci modulating different steps in the EtNU-induced malignant conversion of Schwann precursor cells, but without functional difference between individuals, were sought simultaneously.

We have reported previously (13) that after the initial EtNU-induced T:A→A:T transversion mutation in rat Schwann cell precursors at nucleotide 2012 of the neu gene on chromosome 10, these cells lose heterozygosity for the mutant allele later on in the process of schwannoma development (14, 15). Identification of the mechanism(s) responsible for this LOH by allelic deletion mapping as well as the genetic analysis of schwannoma susceptibility required heterozygous animals. F2 hybrids were generated by crossing inbred BDIX rats (highly sensitive to EtNU-induced schwannoma development) with rats of the resistant inbred BDIV strain. The BDIV alleles exerted a strong inhibitory effect on schwannoma development, as reflected by a markedly reduced tumor incidence and prolonged latency time (Fig. 1). This suppression can be effected either by a dominant resistance gene with decreased penetrance or by polygenic inheritance. The involvement of three independently segregating genes has previously been suggested for the induction of schwannomas by EtNU in sensitive LE and resistant WF rats (28).

One hundred % (17 of 17) of (BDIX × BDIV) F1 schwannomas displayed LOH of varying size on chromosome 10. The smallest region of overlap (Dis1) for the LOH defined by one of the trigeminal schwannomas encompasses the centromeric tip of chromosome 10, beginning with the microsatellite marker D10Rat3 but excluding neu. Dis1 localizes to 25 cM telomeric of Dis1. This indicates that inactivation of a gene(s) in this region is a necessary event for the schwannoma development in heterozygous animals. A strong bias toward losing the BDIV allele was noted.

LOH analyses of EtNU-induced schwannomas of F2 animals necessarily yielded less information because these tumors were not heterozygous for all of the markers used. Chromosome 10 LOH were of different sizes and, when informative, always included the telomeric tip. However, the region of interest became extended because P of 0.005 was obtained for the flanking markers D10Wox23 and D10Mgh5. Combining both results yielded a linkage core region around D10Wox23 with a minimal pointwise P of <0.0005. This locus was called Mss1 (Fig. 2B) for mediating schwannoma susceptibility (see Table 1).
for tumorigenesis in F2 animals, because of either their individual allelic constitution at other loci or different LOH. Alternatively, a small LOH on chromosome 10 may have existed but escaped detection given the marker density available or may have been obscured by contamination of the schwannoma tissue with normal cells.

Judging from the karyotypes recorded in short-term cultures of primary schwannoma cells that exhibited two chromosomes 10 with no apparent deletion or aberration, we could conclude that the likely mechanisms underlying LOH are either uniparental disomy, if the whole chromosome is affected, or mitotic recombination in the case of smaller LOH.

At this stage of analysis, the data seemed to be consistent with the presence of a tumor suppressor gene at the Dis1 locus, which is supposed to be more active within the BDIV than the BDIX allelic configuration. The location of the smallest region of overlap on the tip of chromosome 10 argues against the possibility that the LOH for Dis1 occurred primarily because of selective pressure against the remaining wild-type neu allele in the process of malignant transformation. The heterozygously mutant neu gene seems to be crucial in the initial phase, if not in all of the phases, of EtNU-induced oncogenesis in the PNS, providing mutant Schwann precursor cells with a proliferative advantage and differentiation arrest (14). The observed loss of the wild-type neu allele at later stages of carcinogenesis may be indicative of the loss of a tumor suppressor gene in its proximity rather than of a functional phenomenon.

The demonstration of allele-specific LOH is considered a powerful tool for the detection of functionally polymorphic tumor suppressor genes possibly associated with an inherited predisposition to “sporadic tumors” (29). The LOH on chromosome 10 observed in schwannomas of (BDIX × BDIV) F1 animals pointed to a gene(s) in the telomeric region of this chromosome, and in the great majority of cases, the lost allele originated from the resistant parent. Compared with the sensitive BDIX rats, BDIV rats should thus possess the functionally more active alleles.

Targeted linkage mapping was, therefore, performed by the screening of chromosome 10 with 22 microsatellite markers by a two-step procedure using 78 affected F2 intercross and 55 F2 backcross animals. As recommended by Lander and Kruglyak (30) and Morton (31), we focused on the region showing Ps < 0.05 in the intercross rats and found linkage with a minimum P around D10Wox23 using a second sample of F2 backcross rats. The pointwise Ps obtained by the two-step procedure are <0.005 for D10Wox6 and <0.0005 for ngfr and D10Wox23. The chromosome-wide P is 0.002 (as determined by simulations assuming 20 equally spaced markers, each 5 cM apart; data not shown), which results in a significant genome-wide P of <0.05.

In contrast to our expectations, the Mss1 locus was found to be separated by ~30 cM from the chromosomal area covered by the smallest region of overlap (Dis1) for the LOH. It is noteworthy that in 16 of 17 F1 trigeminal schwannomas, the Mss1 locus was also deleted. Therefore, the loss of Mss1, too, seems to represent a necessary event in schwannoma development, with the allele specificity likely to be relevant for this locus only. Interstitial deletions exclusively covering this region have not yet been found.

A number of well-known tumor suppressor genes such as brca1, nf1, nf2, and p53 are located on rat chromosome 10 (32–34), which shares complete homology with mouse chromosome 11 and, regarding its telomeric half, partial homology with human chromosome 17. However, none of these genes map to the two regions of interest, whereas the neu gene—with a marginal location in the Mss1 region—remains a theoretical but unlikely candidate. Interestingly, Mss1 seems to encompass the region covered by Par1, a locus involved in resistance to urethane-induced lung tumors in Mus spretus (35). The chromosomal region containing Par1 is rich in genes that are critically involved in the control of cell differentiation and proliferation. On the other hand, it remains equally possible that an as-yet-undiscovered “master gene(s)” governs resistance to oncogenesis that is induced by direct-acting carcinogens in different types of target cells.

LOH encompassing Mss1 as well as Dis1 include the brca1 gene. However, a subgroup of human ovarian and breast carcinomas display LOH exclusively at 17q25 (the region homologous to Dis1) leaving brca1 at 17q21 unaffected (36, 37), which underscores the importance of Dis1 for tumor suppression.

Although the biological function of Mss1 is not known at present, it seems unlikely that it is involved in the initial phase of schwannoma development. The diagnostic T:A→A:T transversion at nucleotide 2012 of the neu gene is present in EtNU-exposed trigeminal Schwann precursor cells of the resistant BDIV rats as well as in those of BDIX and (BDIX × BDIV) F1 animals. Heterozygous neu mutant cells at elevated risk of subsequent malignant conversion are thus present in the trigeminal nerves of all of these three animal groups. However, only in the susceptible BDIX strain (and in a small subgroup of F1 animals), do malignant schwannomas actually develop from these cells.7 We assume, therefore, that genetic factors including Mss1 in the BDIV genome prevent initiated Schwann precursors from progressing to the stage of full-blown malignant schwannoma cells. In this scenario, a critical secondary step is obviously strongly favored in F1 animals when initiated cells lose the BDIV-derived Mss1 allele. The involvement of susceptibility genes during later stages of carcinogenesis has also been proposed for other loci predisposing to experimentally induced rodent tumors, such as 1.2-dimethylhydrazine-induced colon tumors (5) and mammary carcinomas induced by N-methyl-N-nitrosourea (38).

It is likely that, in addition to Mss1, other loci contribute to increased susceptibility toward EtNU-induced schwannoma development in the rat. The observed 30% incidence of trigeminal schwannomas among rats of the F2 generation slightly exceeds the 25% incidence expected for a single recessive susceptibility gene and may be an underestimate in view of the concomitant occurrence of other neuroectodermal tumors in these animals. Moreover, the genotype for the linked markers D10Wox6, ngfr, and D10Wox23, as observed in about one-third of schwannomas in F2 backcross animals, is inconsistent with expectations based on the assumption of single-gene susceptibility with no recombination occurring in the candidate region. Instead, several unlinked genes differing functionally between BDIX and BDIV rats might modulate susceptibility or resistance, respectively. A search for such loci by whole genome scanning is in progress.

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GENETIC SUSCEPTIBILITY TO RAT SCHWANNOMA INDUCTION


Ethynitrosourea-induced Development of Malignant Schwannomas in the Rat: Two Distinct Loci on Chromosome 10 Involved in Tumor Susceptibility and Oncogenesis

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