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Genome-wide Screen for Allelic Imbalance in a Mouse Model for Neuroblastoma[1]

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ABSTRACT

We have used the rat tyrosine hydroxylase promotor to overexpress *MYCN* in the neural crest of transgenic mice, resulting in a mouse model for neuroblastoma. Using PCR analysis of microsatellite markers, we conducted a genome-wide analysis in tumors from these animals. Regions of chromosomes 1, 3, 10, 11, 14, and 18 were affected in 20–50% of tumors. Analysis of a subset of these tumors by comparative genomic hybridization was consistent with the microsatellite data. The changes on mouse chromosomes 1, 11, 14, and 18 were syntenic with corresponding regions of loss of heterozygosity in human neuroblastoma, suggesting that genes implicated in the mouse tumors may also play a role in the pathogenesis of the human disease. One-third of the mouse tumors shared abnormalities on chromosomes 1, 3, and 10, whereas the remainder of tumors did not show this combination. These data suggest that genetic mutations on chromosomes 1, 3, and 10 cooperate in the pathogenesis of neuroblastoma and that neuroblastoma in the mouse arises from at least two distinct genetic pathways, one of which is dependent on lesions in chromosomes 1, 3, and 10, the other of which is not.

INTRODUCTION

Neuroblastoma is a tumor of neural crest origin, represents the third most common tumor of childhood, and accounts for 15% of cancer related deaths in children (1). The majority of children with neuroblastoma present with advanced disease, which is generally incurable. Amplification of *MYCN* is the best-characterized genetic abnormality in neuroblastoma and correlates with poor outcome (2).

Analysis of neuroblastoma by LOH[3] or allelic imbalance has identified consistent LOH at chromosomes 1p, 3p, 4p, 11q, and 14q, and gain of chromosome 17q (3–7). Additional LOH was reported on 2q, 9p, and 18q in one study (8). Despite the identification of numerous chromosomal regions that are consistently altered in neuroblastoma, none of the genes corresponding to these regions has been identified to date. A candidate for the gene affected by losses at 1p was recently reported (9), but loss of this gene in neuroblastoma has been disputed (10, 11).

We have developed a transgenic mouse model for neuroblastoma by targeting the expression of *MYCN* to the neural crest of transgenic mice. These animals develop tumors with many features of childhood neuroblastoma (12). Analysis of tumors by CGH demonstrated changes in a number of chromosomal regions that are syntenic with those found in human neuroblastoma. We are therefore using these mice to facilitate the discovery of genes that contribute to the pathogenesis of neuroblastoma.

The ability to physically map regions of chromosomal gain and loss in the mouse has been greatly facilitated by the identification of over 7000 polymorphic markers (13). Most of these are SSLP markers, and the frequency of polymorphism for each marker is in part a function of strain. For crosses between *Mus musculus* mice of strain C57BL/6J and mice of subspecies *M. musculus castaneus*, nearly all of these markers are polymorphic (14).

We have turned to PCR analysis of microsatellite markers for further localization of subchromosomal regions of loss (or gain) in our mouse model of neuroblastoma. PCR analysis of microsatellite markers offers an advantage over CGH in that it can detect smaller regions of loss. In addition, by performing PCR using fluorescently labeled primers for SSLP loci, we can quantitate the extent of allelic gain or loss and control for nonneoplastic contamination of tumor DNA (15). In comparison to CGH and FISH analyses, however, use of fluorescent DNA primers for quantitation of PCR products does not readily distinguish gain from loss (16). The term "allelic imbalance analysis" is used to clarify that this technique can measure either gain or loss of an SSLP marker without distinguishing between the two.

We have crossed animals transgenic for *MYCN* in a C57BL/6J strain with mice of subspecies *castaneus*. Progeny develop neuroblastoma at a frequency of approximately 10%. We have taken 23 tumors from these animals and used SSLP markers to performed a genome-wide screen covering all 19 autosomes and chromosome X. In this communication, we report the allelotype of neuroblastoma in the mouse. Four of six chromosomal regions identified were syntenic with regions of LOH identified in childhood neuroblastoma. In addition, we have identified three chromosomal loci that show common allelic imbalance in a substantial minority of tumors. These data suggest that at least two distinct *MYCN* dependent signaling pathways can lead to neoplastic transformation in the mouse neural crest.

MATERIALS AND METHODS

Mice. The TH-*MYCN* transgenic model has been described previously (12). C57BL/6J and *M. musculus castaneus Ei* mice were from The Jackson Laboratory (Bar Harbor, ME).

Tumor Purification and DNA Isolation. Primary neuroblastomas were dissected free of adherent normal tissues, and tumors frozen in liquid nitrogen for storage. Tumor tissue was minced with a scalpel, and tumors were washed three times in 10 mM Tris, pH 8, 0.1% Triton X-100, 5 mM $MgCl_2$, and 0.32 M sucrose. Pellets were resuspended in 10 mM Tris, pH 8, 200 mM NaCl, 25 mM EDTA, 0.5%SDS, and 0.2 mg/ml proteinase K and incubated at 55°C overnight, followed by phenol extraction and ethanol precipitation.

SSLP Genotyping and Allelic Imbalance Analysis. We examined all 19 autosomes and chromosome X for allelic imbalance in 23 cases of neuroblastoma using 44 markers, representing at least two widely spaced SSLP markers per chromosome. One of the PCR primers for each locus was labeled with fluorochromes Tet or Fam (Research Genetics, Huntsville, AL) to generate a fluorescent PCR product. The SSLP markers used in this study were *D1Mit5, D1Mit200, D2Mit17, D2Mit63, D2Mit151, D3Mit12, D3Mit200, D4Mit138, D4Mit129, D4Mit161, D4Mit60, D5Mit188, D5Mit294, D6Mit138, D6Mit201, D7Mit12, D7Mit246, D8Mit31, D8Mit95, D9Mit262, D9Mit221, D10Mit33, D10Mit44, D11Mit203, D11Mit174, D11Mit36, D11Mit99, D12Mit25, D12Mit10, D13Mit177, D13Mit13, D14Mit105, D14Mit109, D15Mit13, D15Mit175. D15Mit42, D16Mit128, D16Mit181, D17Mit100, D17Mit187, D18Mit70, D18Mit103, D19Mit10, D19Mit80,DXMit18, DXMit154.*

PCR was run using 20 ng of DNA, and reactions were checked to assure that amplification was within the linear range. The PCR products from tumors and

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[3] The abbreviations used are: LOH, loss of heterozygosity; CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; SSLP, simple sequence length polymorphic.

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Chromosome	1		2		3		4		5		6		7		8		9		10		11			12		13		14		15		16		17		18		19		X	
	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	C	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D
Tumor																																									
W						■										I						■						■												M	M
V																								I													■				
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R					■	■									■						■		■																	■	
Q					■	■																					I					I					■			M	M
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C	■				■	■					■	■	■						■					I	I			■	■							I				M	M
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% AI	30%				56%														30%		21%							30%								35%					

Fig. 1. Frequency of allelic imbalance. *Left column,* individual tumors (*denoted by letters A–W*). Numbers in the *top row* correspond to numbered mouse chromosomes and the X chromosome, and *letters P, C,* and *D* in the *second row* indicate the location of SSLP markers at the proximal (*P*), central (*C*), and distal (*D*) regions of the acrocentric mouse chromosomes. *Black boxes* represent allelic imbalance. *I,* indeterminate result. *M,* male mice uninformative for markers on the X chromosome. Numbers in the *bottom row* give the percentage of allelic imbalance for all chromosomes that show greater than 20% allelic imbalance. A subset of tumors (*A–G*) shows consistent allelic imbalance for chromosomes 1, 3, and 10. Four of these seven tumors show additional imbalance for chromosome 6.

spleen controls were run on an ABI 377 fluorescence-based electrophoretic DNA sequencer and analyzed using Genescan software (PE Biosystems, Foster City, CA). We screened for imbalance by measuring the fluorescence intensity of each of the two alleles in multiple spleen DNA controls and calculating the average allele ratio in spleens. A similar fluorescence intensity ratio was then measured in each tumor sample and divided by the average normal ratio to generate an allelic imbalance index. Allelic imbalance was noted when the allelic imbalance index or its inverse was greater than 1.5 (15). Because allelic imbalance is determined using a ratio of allele fluorescence intensity, this method cannot distinguish between loss of one allele and gain of the other.

As a first round of screening, allelic imbalance in C57BL/6J X *castaneus* F1 hybrids was detected by comparing tumor DNAs with three normal spleen DNAs. All chromosomes showing allelic imbalance in more than 20% of tumor samples were then reanalyzed for confirmation of allelic imbalance using the same marker and nine spleen controls. In rare cases of disagreement between the two analyses, allelic imbalance was called only when the allelic imbalance ratio or its inverse was greater than 1.3 for the run not showing allelic imbalance.

CGH. CGH analysis was performed and analyzed as described previously (12). Each tumor was analyzed twice, inverting the red FITC-NEN and green Alexa 568 fluors for the separate analyses.

RESULTS

Generation of Polymorphic Tumors. Mice transgenic for *MYCN* were originally made by injecting the TH-*MYCN* transgene into eggs from out-crossed F1 mice of strains C57BL/6J and BALB/c (12). A resulting transgenic founder containing four copies of the *MYCN* transgene was serially back-crossed to the C57BL/6J parental strain for seven generations (12).[4] F7 female mice were then crossed to *castaneus* males, as the *castaneus* females rarely would nurse their pups. In addition, crosses were regularly updated with transgenic mice that had been back-crossed further to C57BL/6J, so that the C57BL/6J parents for the mice in this study represented F7-F10 animals with respect to serial back-crossing to C57BL6/J.

Mice were euthanized when tumors were visible and were occasionally found dead from tumor. Histological analyses of tumors were performed in all cases (data not shown) and were similar to those reported previously (12). Tumors were found in abdominal or thoracic paraspinous locations, were seen only during the first year of life in the mouse, and stained positively for the neuronal markers synaptophysin and neuron-specific enolase (12).[4]

We followed approximately 250 transgenic animals derived from a cross between transgene-positive C57BL/6J females and males of the substrain *castaneus*. Latency for tumors varied from 3 to 12 months, with penetrance of approximately 10% by 1 year. No additional tumors were seen after 1 year of age. These survival data are consistent with observations that penetrance in mice transgenic for *MYCN* showed steady decline with increasing serial back-crosses to C57BL/6J (data not shown). The decreased penetrance of tumors in strain C57BL6/J stands in marked contrast to the increased penetrance of neuroblastoma observed during serial back-cross to mice of strain 129Sv/J (12).[4]

Incidence and Pattern of Allelic Imbalance in Mouse Neuroblastoma. We examined 23 cases of neuroblastoma using 46 markers, representing at least two widely spaced SSLP markers per chromosome (see "Materials and Methods" for details). The percentage of mice informative at each locus ranged from 80 to 100%. Information was therefore available from 18–23 animals per locus and at 38–44 loci per tumor.

The incidence of allelic imbalance ranged from 0 to 56% at each of the 20 chromosomes examined and averaged 15%. The number of

[4] W. A. Weiss and J. M. Bishop, unpublished data.

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Chromosome		I		II		III		IV		V		VI		VII		VIII		IX		X	
		P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D
Tumor																					
G	AI	■				■	■	■												■	
G	CGH	G	G			G	G														
F	AI	■	■			■	■					■	■			■	■			■	
F	CGH	G	G			G	G			L	L	G	G	L							
D	AI	■		■	■	■	■	■				■								■	
D	CGH	G	G	G	G	G	G							L							

Chromosome		XI			XII		XIII		XIV		XV		XVI		XVII		XVIII		XIX		X	
		P	M	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D	P	D
Tumor																						
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Fig. 2. CGH analysis for three tumors and comparison with allelic imbalance data. CGH was performed on three tumors (see "Materials and Methods" for details). *G,* gain; *L,* loss. All gains and losses involved whole chromosome changes except for chromosome 7, which showed loss of the proximal one-third only. Metaphase spreads used for CGH analysis were mismatched on the X chromosome, so this chromosome could not be evaluated by CGH.

allelic imbalances per tumor varied from 0 to 10 and averaged 3.5. Fig. 1 demonstrates that allelic imbalance above 20% was seen at chromosomes 1 (30%), 3 (56%), 10 (30%), 11 (21%), 14 (30%), and 18 (35%). Allelic imbalance in the remaining 14 chromosomes was less than 10%.

Approximately one-third of tumors showed allelic imbalance on the proximal region of chromosome 10. For chromosomes 3 and 11, most affected tumors showed involvement of all markers tested. A mixed pattern of partial and whole chromosome allelic imbalance was seen on chromosomes 1, 14, and 18. Tumors with localized changes on chromosome 14 showed abnormalities in either the proximal or distal marker, suggesting a central region of abnormality. Tumors with localized imbalance of SSLP markers on chromosome 1 showed involvement of the proximal marker. In contrast, tumors with localized changes on chromosome 18 showed involvement of the distal marker. Seven tumors showed consistent combinations of imbalances involving markers on chromosomes 1, 3, and 10 (Fig. 1).

CGH Analysis of a Subset of Tumors Demonstrates Gain on Chromosomes 1, 3, and 11. To further validate our allelic imbalance results, we performed CGH analysis on three tumors that showed representative allelic imbalances (Fig. 2). For these tumors, CGH analysis was in general agreement with allelic imbalance analysis for chromosomes 1, 3, and 11. These results confirm that allelic imbalance and CGH analyses of the same tumor sets could identify many of the same lesions.

Correlation of Allelic Imbalance Studies between Mouse and Human Neuroblastoma. Table 1 compares allelic imbalance in mouse neuroblastoma with published LOH and allelic imbalance analyses from human neuroblastoma. When the results of numerous analyses of childhood neuroblastoma were compiled, LOH was seen commonly in eight chromosomal regions, and allelic gain (of chromosome 17q) was seen in one region. Four of these nine regions are syntenic with regions of mouse chromosomes 1, 11, 14, and 18, all of which showed frequent allelic imbalance in this study. Our limited CGH data suggest that regions of mouse chromosomes 1 and 11 show allelic gain in the murine tumors. The syntenic regions of human chromosomes 2q and 17q showed allelic loss and gain respectively. Although LOH at chromosome 2q appears incongruous with our observations of likely gain in syntenic regions of mouse chromosome 1, four CGH studies all demonstrated gain of chromosome 2 in childhood neuroblastoma (17–20).

In childhood neuroblastoma, LOH at chromosome 1p36 and allelic gain at chromosome 17q correlate with amplification of *MYCN* (3, 6, 21). Although we observed allelic imbalance on mouse chromosome 11 (syntenic with 17q), we did not see abnormalities on mouse chromosome 4 (syntenic with human chromosome 1p). The SSLP markers *D4Mit129, D4Mit161,* and *D4Mit60* map at 79.5–81 cM along mouse chromosome 4 in regions of synteny with human chromosome 1p36 (14). One of these markers (*D4MIT161*) is the distal marker shown in Fig. 1. These three markers showed less than 5% allelic imbalance in the 23 tumors tested (data for *D4Mit129* and *D4Mit60* not shown).

Table 1 *Allelic imbalance in human and mouse neuroblastoma*

Numbers in parentheses correspond to appropriate Refs. for human LOH data. Syntenic regions of the mouse genome are from Ref. 14. Boldface regions show common allelic imbalance between mouse and human neuroblastoma. In human neuroblastoma, LOH for 1p is correlated with amplification of *MYCN* (3, 6). Allelic gain for 17q did not correlate with amplification of *MYCN* in one study (7); however, gain of chromosome 17q as measured by CGH or FISH has been correlated with amplification of *MYCN* in a larger study (21).

Human chromosome	LOH (%)	Syntenic mouse chromosome	Allelic imbalance (%)
1p36 (3)	26	4	<10
2q33 (8)	**30**	**1**	**30**
3p (4)	16	6, 9	<10
4p (5)	23	5	<10
9p21 (8)	36	4	<10
11q13 (8)	24	19	<10
14q (6)	**23**	**14**	**43**
17q (7)	**38**	**11**	**21**
18q21 (8)	**31**	**18**	**30**

DISCUSSION

Common Imbalance for Chromosomes 1, 3, and 10 in a Subset of Tumors. These experiments identify six chromosomal regions that show frequent allelic imbalance in tumors from a mouse model of childhood neuroblastoma. Seven tumors showed consistent combinations of imbalances involving markers on chromosomes 1, 3, and 10 (Fig. 1). A subset of these also showed an imbalance involving chromosome 6. The tumors showing changes on chromosomes 1, 3, and 10 arose with an average latency of 7 months, compared with 4 months for those without involvement of chromosomes 1, 3, and 10. Histopathological analysis revealed no obvious difference between tumors with and without involvement of these chromosomal regions.

The association of imbalances on chromosomes 1, 3, and 10 suggests that specific combinations of chromosomal lesions occur commonly in mouse neuroblastoma, that these combinations of lesions may interact to contribute to neuroblastoma in the mouse, and that allelic imbalances for chromosomes 1, 3, and 10 represent part of a pathway that contributes to transformation in the neural crest. Specific combinations of genetic lesions have been described in colon cancer, for example, in which mutations in *APC*, *K-RAS*, *p53*, and a tumor suppressor on chromosome 18q21 are found together in this malignancy (22). We have not analyzed metaphase spreads from these tumors, however, and it is possible that a consistent unbalanced translocation could explain the association of imbalances between two specific chromosomes.

The F1 offspring used in this study were derived from transgene-positive C57BL/6 females crossed with *castaneus* males and were essentially genetically identical to each other. It is therefore surprising that a subset of tumors from these animals shows allelic imbalance

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among alleles on chromosomes 1, 3, and 10, whereas this association was not observed in the remaining tumors. These data suggest that at least two distinct pathways may be used in *MYCN* dependent transformation of mouse neural crest (one of which involves chromosomes 1, 3, and 10) and an alternate pathway(s) that is not dependent on loci on these chromosomes.

Correlation of CGH with LOH in Mouse Neuroblastoma. We had previously analyzed a different set of 21 tumors by CGH to screen for consistent copy number abnormalities (12). The tumors from the previous study were all derived from mice of mixed C57BL/6J and BALB/c strain backgrounds, unlike the tumors in this study that arose in F1 offspring from a C57BL/6J X *castaneus* cross. The present allelic imbalance and previous CGH studies both showed abnormalities in chromosomes 3 and 11 but were not in agreement for other chromosomal regions. Technical differences between CGH and allelic imbalance, the fact that tumors from mice of different strain backgrounds were used in the two studies, and the relatively small sample sizes all are likely contributors to the apparent discrepancies between the two studies. Because all mice in this study were generated by crossing a *castaneus* male with a transgene positive female, it is formally possible that imprinting could underlie some of the apparent differences between these two studies. An imprinted (transcriptionally silenced) tumor suppressor gene might not undergo somatic mutation in these animals, whereas the same gene might be mutated in a cross between transgene positive C57BL/6J males and *castaneus* females.

To evaluate whether CGH and allelic imbalance analyses could yield comparable results in our hands, we performed CGH analysis on three tumors from this study that were also analyzed by allelic imbalance. We chose tumors that among them showed allelic imbalance on chromosomes 1, 3, 10, 11, 14, and 18. The results demonstrated that both techniques were generally consistent in picking up changes on chromosomes 1, 3, and 11, which CGH analysis identified as chromosomal gains. CGH did not detect the allelic imbalance on chromosome 10, which was present in all three tumors. We have recently localized this region to lie within a 15-cM interval,[5] and it is likely that CGH would not reliably identify a localized region of gain or loss.

We observed regions of allelic imbalance not seen by CGH and CGH changes that were not identified in the allelic imbalance study. This apparent discrepancy may reflect the limits of resolution for CGH, or it could in part result from the poor sensitivity of CGH in analyzing chromosomes near the centromeres, where some of our SSLP markers mapped.

These differences may also reflect differences in the way in which CGH and allelic imbalance studies measure abnormalities. Mechanisms of subchromosomal allelic imbalance include deletion, duplication, mitotic recombination, nondisjunctional chromosome gain, nondisjunctional chromosome loss with or without reduplication, locus-restricted events such as gene conversion or point mutation, and epigenetic allelic inactivation (23). In a locus that becomes homozygous through mitotic recombination, gene conversion, or chromosome loss with reduplication, allelic imbalance analysis would detect this result whereas CGH would not. Similarly, in an aneuploid tumor, CGH may score a gain or loss that restores the ratio of maternal to paternal alleles in that region, so no allelic imbalance would be seen.

Comparison to Human Tumors. Four of the six regions displaying allelic imbalance in tumors from this study were syntenic with regions of LOH or allelic gain in published studies of human neuroblastoma (Table 1). We could evaluate the congruence of these changes for two of the four mouse regions. Our limited CGH analyses suggest that the allelic imbalance for chromosomes 1 and 11 may represent gains. Mouse chromosomes 1 and 11 are syntenic with human chromosomes 2 and 17 respectively, both of which show gain in CGH analyses of childhood neuroblastoma (14, 17–20). The gain on human chromosome 2 stands in contrast to a report of LOH corresponding to this region (8); however, the CGH result has been observed in four separate studies (17–20). LOH analysis of human chromosome 17 was consistent with the CGH result and demonstrated allelic gain (7). Therefore, for two regions in which we can evaluate congruence, the human and mouse data are in likely agreement.

CGH and FISH analysis of childhood neuroblastoma showed gain of chromosome 17q to correlate with loss of loss of 1p and with amplification of *MYCN* (21). In agreement with observations in childhood neuroblastoma, we observed likely gain of mouse chromosome 11 (syntenic with human 17q).

Loss of material at human chromosome 1p36.2 correlated with amplification of *MYCN* in childhood neuroblastoma (3, 6). However, in our analysis of three markers on mouse chromosome 4 that map to regions syntenic with human chromosome 1p36, we did not observe significant allelic imbalance. It is possible that loss of a presumed tumor suppressor on human chromosome 1p36 is required to render the genome unstable and allow for subsequent amplification of *MYCN*. Because tumors in our mice are initiated by an overexpressed transgene of *MYCN*, there may be no requirement for amplification of the gene, thus bypassing the need for deletion of a tumor suppressor on 1p36. In addition, there is some controversy regarding the location of this tumor suppressor and whether one or two tumor suppressors lie in this region of chromosome 1p (24–26). If the tumor suppressor on 1p36 lies outside of the region of synteny analyzed in the present study, it may have escaped detection.

The synteny between four regions commonly altered in both mouse and human tumors suggests that the mouse model is a faithful representation of human neuroblastoma and that further localization of these regions in the mouse may identify genes of importance to childhood neuroblastoma as well.

[5] W. A. Weiss, unpublished data.

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