Targeted Disruption of the 3p12 Gene, Duttil/Robo1, Predisposes Mice to Lung Adenocarcinomas and Lymphomas with Methylation of the Gene Promoter

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ABSTRACT

The DUTT1 gene is located on human chromosome 3, band p12, within a region of nested homozygous deletions in breast and lung tumors. It is therefore a candidate tumor suppressor gene in humans and is the homologue (ROBO1) of the Drosophila axonal guidance receptor gene, Roundabout. We have shown previously that mice with a targeted homozygous deletion within the Duttil/Robo1 gene generally die at birth due to incomplete lung development: survivors die within the first year of life with epithelial bronchial hyperplasia as a common feature. Because Duttil/Robo1 heterozygous mice develop normally, we have determined their tumor susceptibility. Mice with a targeted deletion within one Duttil/Robo1 allele spontaneously develop lymphomas and carcinomas in their second year of life with a 3-fold increase in incidence compared with controls: invasive lung adenocarcinomas are by far the predominant carcinoma. In addition to the mutant allele, loss of heterozygosity analysis indicates that these tumors retain the structurally normal allele but with substantial methylation of the gene’s promoter. Substantial reduction of Duttil/Robo1 protein expression in tumors is observed by Western blotting and immunohistochemistry. This suggests that Duttil/Robo1 is a classic tumor suppressor gene requiring inactivation of both alleles to elicit tumorigenesis in these mice.

INTRODUCTION

The identification of genes associated with consistent tumor-specific chromosomal abnormalities has been an effective method of isolating genes important in cancer development (1). Deletion mapping of chromosome 3 has indicated that somatic genetic loss to the short arm is a frequent event in a variety of common carcinomas and raised the expectation that tumor suppressor genes will be found located within the minimally deleted regions (2). Lung cancer has received the most attention because many studies have indicated a very high incidence of loss on 3p (91% for small-cell lung carcinoma, 95% for squamous cell lung carcinoma, and 71% for adenocarcinoma) and because 3p loss is detectable in low-grade preinvasive lesions, implying an early, possibly initiating, role for 3p loss in lung tumorigenesis (3). Several distinct regions along the chromosome have been delineated, and their boundaries have been reduced by the discovery of homozygous deletions nesting within the region of heterozygous loss (2). A number of genes within these deletions have been examined for frequent point mutations in tumors, but none has shown this classical feature of tumor suppressor genes (4, 5), although reduced expression due to promoter hypermethylation is common (6).

Our search for a tumor suppressor gene has focused on proximal 3p and identified three nested homozygous deletions, one of which is within a gene, DUTT1 (deleted in U-twenty-twenty; ref. 7). This intragenic deletion removes a segment encompassing exon 2 of the gene, creating a mutant form of the protein lacking the first immunoglobulin domain. The DUTT1 gene was independently isolated as the human homologue (ROBO1) of the Drosophila gene Roundabout (Robo; ref. 8). Duttil/Robo1 is a trans-membrane receptor responding to the Slit family of ligands and mediates signals that control cell migration and cell position in a range of cell types from Drosophila to mammals (9). It is not known whether it performs a similar role in epithelial cells, but abrogation of this function in tumors could explain the contribution of DUTT1/ROBO1 to malignancy because inappropriate migration and incorrect cell position are such defining features of the malignant phenotype (10). However, although positional clonning indicates that DUTT1/ROBO1 is a candidate tumor suppressor gene, like other candidates on 3p, tumor-associated point mutations have not been detected (11). If the mechanism of DUTT1/ROBO1 gene inactivation is epigenetic, a functional demonstration is required to conclusively confirm its involvement in tumor development. Tumor formation in mice with targeted inactivation of the candidate gene is recognized to fulfill this requirement (12).

To investigate the possibility that inactivation of this gene predisposes to tumor development, we generated a targeted intragenic deletion within the Duttil/Robo1 gene in the mouse germ line, recapitulating the loss of exon 2 detected in a human small-cell lung carcinoma (13). Frequent perinatal mortality is observed in mice homozygous for the disrupted Duttil/Robo1 gene. The few surviving homozygous mice die within 12 months, having developed extensive bronchial hyperplasia. We now demonstrate that heterozygous mice, with this deletion within only one allele, spontaneously develop lymphomas and carcinomas in their second year of life: invasive carcinoma of the lung is by far the predominant epithelial tumor. Duttil/Robo1 expression is substantially reduced in these tumors and is accompanied by substantial methylation of the gene’s promoter. We conclude that Duttil/Robo1 acts as a classic tumor suppressor gene with inactivation of both alleles in the development of malignant tumors in these mice.

MATERIALS AND METHODS

Observation of Abnormal Mice Phenotypes. The Duttil/Robo1 mutant mice (mixed C57Bl/6 and 129/Sv genetic background) were generated by gene targeting (13). Duttil/Robo1 genotype was determined by standard Southern blot hybridization analysis of tail DNA as described previously (13). The mice were housed in a pathogen-free environment, and their care was subject to United Kingdom Home Office regulations. They were observed frequently and sacrificed on decline in their health or obvious tumor burden. Complete necropsy was carried out on all mice. Histopathological examination was performed on all tissues found with visible abnormalities in mutant mice and controls. Tissues were fixed in buffered 4% paraformaldehyde and then embedded in paraffin wax, and the wax blocks were sectioned at 5 to 8 μm and stained with hematoxylin and eosin (H&E). Large tumors were also frozen for extraction of DNA, RNA, and protein using standard procedures.

Southern Blotting. Genotyping of tumors was performed as described previously for genotyping of tail DNA (13).
Reverse Transcription-Polymerase Chain Reaction. Total RNA was prepared from mouse tumors, fibroblasts, and normal lung with Trizol reagent (GIBCO/BRL, Gaithersburg, MD). Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously (13). Mouse Gapdh mRNA was amplified as a template control. For polymerase chain reaction (PCR) of Dutt1/Robo1, the primers used were as follows: (a) exon 1 forward (5'-AGGCGTTAAGGACACTCCCG-3') and exon 2 reverse (5'-AGTACT-CCTCAGCGAGGTGG-3'); this PCR fragment includes exon 1 and exon 2 (527 bp); and (b) exon 3 forward (5'-ATACTACGGATGACTTCAG-3') and exon 5 reverse (5'-CTGGATTTGGCAGCTTCC-3'); this PCR fragment includes exon 3 to exon 5 (230 bp). For PCR of mouse Gapdh mRNA, the primers used were as follows: forward, 5'-TGAACTGGATCGGCG-TATT-3'; and reverse, 5'-TGCCGTGAGTGAGTCATAC-3'. The products were visualized by ethidium bromide staining after electrophoresis on agarose gels.

Bisulfite Modification and Methylation Analysis. Bisulfite sequence analysis was performed as described previously (14). One microgram of genomic DNA from normal or tumor tissues in a volume of 50 μL was denatured in 0.2 mol/L NaOH for 15 minutes at 37°C. Thirty microliters of 10 mmol/L hydroquinone (Sigma, St. Louis, MO) were added to each sample, mixed gently, and left at room temperature for 5 minutes; 520 μL of freshly prepared 3 mol/L sodium bisulfite (pH 5; Sigma) were added; and samples were incubated under mineral oil at 50°C for 16 hours. Modified DNA was purified using Wizard DNA purification resin according to the manufacturer's instructions (Promega, Madison, WI) and eluted with 60 μL of water. Methylation analysis was completed by incubation with NaOH to 0.25 mol/L for 15 minutes at 37°C, followed by ethanol precipitation. DNA was resuspended in Tris-EDTA and used immediately or stored at −20°C.

Bisulfite-modified DNA (100 ng) was amplified using the mouse Dutt1/Robo1 promoter region-specific primers designed by the MethPrimer program. The promoter region was amplified in two parts. CpG island region 1 from position −793 to −616 was amplified using forward primer 5'-GAAGTTGGTTTGTATTGTGTTGGT-3' and reverse primer 5'-AACCTTTATTCCACATC-3'. CpG island region 2 from position −443 to the beginning of exon 1 (position −310) was amplified using forward primer 5'-GAGGAGTATTTTTGTGGGTTG-3' and reverse primer 5'-TTTCTACTACCTCCACATC-3'. PCR conditions were 95°C for 12 minutes and 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. PCR products were purified using QIAquick PCR purification kit (Qiagen). Methylated cytosine residues were identified by direct sequencing of PCR products with the same primers used for PCR.

Protein Detection by Western Blotting and Immunohistochemistry. Immunohistochemical staining with a polyclonal antibody raised against the COOH-terminal peptide of DUTT1/ROBO1 (CYERGEDNNEEEL) was performed as described previously (15). Western blotting, using the same antibody, was as described previously (13).

RESULTS

Tumor Incidence in Dutt1/Robo1 Heterozygous Mice. Dutt1/Robo1+/− mice die at birth or within the first year of life, on average at 7 months, preventing evaluation of the effect of the homozygous deletion within this gene, which results in a truncated protein, on tumor development (13). To assess whether tumors develop in heterozygotes, a cohort of 154 Dutt1/Robo1+/− mice was aged together with 106 wild-type littermates. Eight control mice (7.5%) developed tumors (three mice developed lung tumors, and five mice developed lymphomas), on average at 20.2 months. The incidence of lung tumors (2.8%) and lymphomas (5%) is within the range expected from the genetic background of the mouse used in these studies.4 In the Dutt1/Robo1+/− group, 34 mice (22.0%) developed a total of 53 tumors (Fig. 1A). This is a significant increase in tumor predisposition compared with wild-type mice; (P < 0.01, χ² test). Other than lymphomas, the tumors (both benign and malignant) were almost all epithelial in origin (Fig. 1B).


The germ-line disruption in mice of other tumor suppressor genes associated with lung cancer development, such as p53, p16INK4a, and Rb, does not show increased incidence of lung cancer development (16). In contrast, 26.5% of the tumors that developed in the Dutt1/Robo1+/− mice were adenocarcinomas of the lung (Fig. 1B). Their development was spontaneous and not dependent on any guidance of genetic damage to the bronchial epithelium.

Phenotypic Characteristics of Lung Tumors Developing in Dutt1/Robo1+/− Mice. The lung tumors that developed in the Dutt1/Robo1+/− mice were all adenocarcinomas, ranged in size from 2 to 10 mm², and generally had an irregular, distorted appearance (Fig. 2A) and showed clear invasion (Fig. 2B) with pleomorphic nuclei (Fig. 2C). Histologic subtypes were typical, papillary, and glandular (data not shown). In contrast, the three lung tumors arising in wild-type mice were very similar to each other and were small (<2 mm²) with a regular pebble-like appearance (Fig. 2D), well circumscribed (Fig. 2E), and with near normal nuclear morphology (Fig. 2F). Direct comparison of tumor incidence (Fig. 1A) in wild-type and mutant mice masks these important differences in the degree of malignancy observed in the lung tumors. Although invasion was clearly detected in the lung adenocarcinomas that developed in the Dutt1/Robo1+/− mice (Fig. 2B), secondary deposits were not observed macroscopically. Examples of the other malignant tumors and preinvasive lesions occurring in the Dutt1/Robo1+/− group are illustrated in Fig. 2G–N.
Dutt1/Robo1 Expression in Tumors from Dutt1/Robo1<sup>+/−</sup> Mice. Because the mutant allele is an intragenic deletion, transcription could occur from both mutant and wild-type alleles (Fig. 3A and B). Primers for RT-PCR were used to detect transcription from either allele (exons 3–5) and specifically from the wild-type allele (exons 1–2; Fig. 3B). Although transcripts were detectable when mouse lung from wild-type mice was the source of template RNA (Fig. 3C, Lane 1), products were barely detectable when RNA from the Dutt1/

Fig. 3. Partial structure and expression of wild-type and mutant Dutt1/Robo1 gene. A, wild-type allele exons 1 to 5 and mutant allele with exon 2 replaced by a neo construct (13). B, the transcripts that arise from the mutant and wild-type genes and the priming sites and PCR products that distinguish the transcripts. C, examination of expression of Dutt1/Robo1 alleles in mutant mice and controls. RT-PCR analysis of RNA from lung tumors and lymphomas amplified with primers from exons 1 and 2 (only RNA from the wild-type Dutt1/Robo1 allele can act as a template) and from exons 3 and 5 (RNA from both wild-type and mutant Dutt1/Robo1 alleles can act as a template). Amplification with primers from Gapdh acts as a template control. Lane 1, RNA from normal lung from a wild-type littermate; Lane 2, RNA from uninvolved lung of Dutt1/Robo1<sup>+/−</sup> mice; Lanes 3–6, RNA from lung tumors; Lanes 7–10, RNA from lymphomas; Lane 11, RNA from fibroblasts from Dutt1/Robo1 homozygous mice lacking exon 2; Lane 12, no template control.
Robo1\textsuperscript{+/-} tumors was used as template (Fig. 3C). In parallel with apparent low transcription levels of the Dutt1/Robo1 gene, using immunohistochemistry, very low levels of Dutt1/Robo1 protein were detected in 9 of 12 lung tumors examined that developed in Dutt1/Robo1\textsuperscript{+/-} mice in contrast to surrounding normal tissue (Fig. 4A and B) and to normal bronchial epithelium (Fig. 4C). Dutt1/Robo1 protein was also virtually undetectable by immunohistochemistry in the three other carcinomas listed in Fig. 1B (data not shown) and in 12 of 17 lymphomas tested (data not shown), but the benign neoplasms tested showed positive staining (Fig. 4D–F), implying that inactivation was associated with the malignant phenotype. The low levels of Dutt1/Robo1 protein in the malignant tumors (lymphomas and lung adenocarcinomas) were confirmed by Western blotting using mouse brain and a human tumor cell line as positive controls (Fig. 4G). Nondissociated tumor samples contain variable amounts of normal cells (e.g., blood vessels, stroma, lymphocytes, and so forth) that may express Dutt1/Robo1 protein, presumably resulting in the trace of protein detectable by Western blotting (Fig. 4G, Lanes 4 and 8).

**Dutt1/Robo1 Gene Structure in Tumors from Dutt1/Robo1\textsuperscript{+/-} Mice.** The RNA and protein expression data suggested that Dutt1/Robo1 gene silencing had occurred in the etiology of the malignant tumors. When tumors develop in mice with targeted disruption of a tumor suppressor gene, the remaining allele is often lost or inactivated, mimicking the behavior of tumor suppressor genes in spontaneously arising human tumors. However, Southern blotting of DNA from lung tumors and lymphomas showed that the wild-type allele was retained in tumors developing in these Dutt1/Robo1 heterozygous mice (Fig. 5A).

To determine whether this apparent gene silencing was linked to hypermethylation of the promoter region of the Dutt1/Robo1 gene, we identified the related CpG islands by referring to the location of those associated with the human DUTT1/ROBO1 gene (11). DNA from lymphomas, lung adenocarcinomas, and controls was treated with sodium bisulfite and amplified with primers flanking two potentially methylated regions (Fig. 5B), and the PCR products were sequenced. DNA from normal lung was unmethylated, as was that from a benign tumor control. In contrast, DNA from the six lymphomas and six lung tumors assessed for methylation of the Dutt1/Robo1 promoter region was all found to be methylated (Fig. 5C). Because the antibody to the COOH-terminal peptide detects both mutant and wild-type protein, we conclude that the very low levels of any Dutt1/Robo1 protein in those malignant tumors tested imply that both alleles are methylated in the malignant tumors of Dutt1/Robo1\textsuperscript{+/-} mice.

**DISCUSSION**

Before the observations described here, only circumstantial evidence linked the human DUTT1/ROBO1 gene to tumor formation (7, 11). In our earlier work, we used gene targeting in mice to recapitulate a specific human intragenic deletion that occurs within the DUTT1/ROBO1 gene. The abnormal phenotypes that developed in mice homozygous for the deletion provided a direct link between this gene and epithelial lung abnormalities in both developing and adult mice (13). In this study, we demonstrate that tumors arise in the heterozygous mutant mice as a direct consequence of the specific genetic disruption of the Dutt1/Robo1 gene, providing the first unequivocal demonstration of a functional association between this candidate tumor suppressor gene and tumor formation. No other candidate tumor suppressor gene mapping within regions of homozygous deletion on 3p has been directly linked in this way to spontaneous epithelial tumor development.

Further support for the Dutt1/Robo1 gene as a tumor suppressor gene is provided by comparison with HIC1 (17). After confirmation of HIC1 as a tumor suppressor gene, not only by the observation of
tumor formation in mice heterozygous for a targeted deletion of Hic1, but also by virtue of methylation of its promoter in these mouse tumors, Chen et al. (18) have proposed that the detection of methylation arising in the course of tumor development in genetically modified mice provides a "prime strategy" for confirming tumor suppressor gene status on genes without somatic mutations as a defining feature.

However, although we believe we have provided compelling evidence that Dutt1/Robo1 behaves as a tumor suppressor gene in the development of these mouse tumors, we cannot at present extrapolate these observations directly to human lung carcinomas because Northern blotting has shown that the DUTT1/ROBO1 gene is expressed in a variety of human tumor cell lines (19). Furthermore, protein is easily detectable by immunohistochemistry in tissue sections of primary human tumors.5 The function of DUTT1/ROBO1 in normal epithelial cells is not known, making it currently impossible to investigate whether the DUTT1/ROBO1 protein detected in human tumors is performing some aberrant function. The apparent disparity between the observed DUTT1/ROBO1 protein expression in these experimental mouse tumors and human tumors may be accounted for by reduced or absent expression at the early stage of lung tumor development and increased expression at a later stage of malignancy, possibly performing some oncogenic function. Transforming growth factor /H9252, which suppresses the early stages of tumorigenesis but promotes tumor progression and invasion, is an example of such a dual function (20). The expression pattern of E-cadherin in tumors also has similar parallels to DUTT1/ROBO1. Loss of E-CAD expression is associated with promotion of invasion, but subsequent reexpression of E-CAD may encourage survival of metastatic deposits. In a study of heterogeneity of expression of this gene in breast cancer, the fluctuation was shown to be modulated by variation in methylation of its promoter (21). During the active selection processes that are part of tumor development, cells with partial reactivation of tumor suppressor genes may be selected because of the growth advantage conferred by reexpression of the gene. For genes with pleiomorphic and opposing functions at different stages of tumor progression, inactivation by an epigenetic process rather than mutation provides a selective advantage during tumor development because reactivation is possible.

5 G. Corbett, J. Xian, M. Arends, J. Catto, and P. Rabbitts, unpublished observations.
SLIT2, a likely ligand for DUTT1/ROBO1, also appears to have both tumor-suppressing and -promoting roles. Dallol et al. (22, 23) consider the SLIT2 gene to be an excellent candidate tumor suppressor due to its growth-suppressing properties and frequent methylation in several human tumors including colorectal, lung, and breast carcinomas. In contrast, Wang et al. (24) detected SLIT2 expression in a wide variety of tumor cell lines and primary tumors and showed in a series of colon preinvasive lesions of increasing grade that the degree of SLIT2 expression correlated with the grade of malignancy, consistent with tumor-promoting properties. Clearly, the roles of DUTT1/ROBO1 and its ligand(s) in tumorigenesis require further detailed investigation, but these observations, together with this report, suggest that a new signaling pathway in tumorigenesis has been identified. Because signaling mediated by this pathway is likely to affect cell motility, an understanding of the abrogation of this pathway in tumorigenesis may provide insight into the mechanisms of tumor invasion and metastatic spread.

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REFERENCES

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