Recessive Transmission of a Multiple Endocrine Neoplasia Syndrome in the Rat[1]

Andreas Fritz, Axel Walch, Kamilla Piotrowska, Michael Rosemann, Ekkehard Schäffer, Karin Weber, Andreas Timper, Gerhild Wildner, Jochen Graw, Heinz Höfler, and Michael J. Atkinson[2]

Institute of Pathology [A. F., A. W., K. P., M. R., E. S., A. T., H. H., M. J. A.] and Institute of Mammalian Genetics [J. G.], GSF-National Research Center for Environment and Health, D85764 Neuherberg; Institute of Pathology, Technical University, D81675 Munich, [A. W., H. H.]; Institute of Animal Physiology, Ludwig-Maximilians-University, D80539 Munich [K. W.]; and Department of Ophthalmology, Section of Immunobiology, Ludwig-Maximilians-University, D80336 Munich [G. W.], Germany

ABSTRACT

We describe a novel hereditary cancer syndrome in the rat that is transmitted by a recessive gene mutation. Animals exhibiting the mutant phenotype develop multiple neuroendocrine malignancies within the first year of life. The endocrine neoplasia is characterized by bilateral adrenal pheochromocytoma, multiple extra-adrenal pheochromocytoma, bilateral medullary thyroid cell neoplasia, bilateral parathyroid hyperplasia, and pituitary adenoma. The appearance of neoplastic disease is preceded by the development of bilateral juvenile cataracts. Although the spectrum of affected tissues is reminiscent of human forms of multiple endocrine neoplasia (MEN), no germ-line mutations were detected in the *Ret* or *Menin* genes that are responsible for the dominantly inherited MEN syndromes in humans. Segregation studies in F1 and F2 crosses yielded frequencies of affected animals entirely consistent with a recessive autosomal mode of inheritance. The lack of the phenotype in F1 animals effectively excludes a germ-line tumor suppressor gene mutation as the causal event. The absence of mutation of known MEN genes and the unique constellation of affected tissues, plus the recessive mode of inheritance, lead us to conclude that the mutation of an as yet unknown gene is responsible for this syndrome of inherited neuroendocrine cancer.

INTRODUCTION

MEN[3] syndromes are rare autosomally dominant genetic diseases in humans. The MEN1 syndrome (OMIM 131100), typically presenting with neoplastic involvement of the parathyroid, endocrine pancreas, and pituitary, is the result of germ-line mutation of the *MEN1* tumor suppressor gene encoding the menin protein, located on chromosome 11q13 (1, 2). Both MEN2A (OMIM 171400), typified by medullary thyroid carcinoma, pheochromocytoma, and neoplasia of the parathyroid gland, and MEN2B (OMIM 162300), presenting with MEN2A features plus mucosal neuromas and a marfanoid habitus, result from mutation of the *RET* proto-oncogene on chromosome 10q11.2. The differences between the two MEN2 phenotypes appear to be the result of functionally distinct *RET* mutations (3–5). The involvement of other genes in MEN2-like syndromes cannot be discounted, because *RET* mutations are absent in a few cases (6). We report here the first description of an MEN-like syndrome in the rat that does not involve mutation of menin or RET. Unlike the MEN syndromes in humans, this endocrine neoplasia syndrome in the rat is the result of the germ-line transmission of a recessive gene mutation.

MATERIALS AND METHODS

Experimental Animals. The MEN-like phenotype was initially identified in a Sprague Dawley rat-breeding colony and subsequently maintained by matings between affected and nonaffected littermates. Matings of affected animals were unproductive because of low fertility, in part because of a lack of ovarian development.[4] Animals were housed according to national laboratory animal welfare regulations and were examined 5 days a week. Visibly sick or moribund animals were euthanized and autopsied. Because cataracts are the first visible sign of the phenotype it was provisionally designated as "Sprague Dawley white eye." The abbreviation SD^{we} is used to denote animals expressing the mutant phenotype. Wild-type Sprague Dawley and Wistar animals for breeding experiments and molecular analysis were obtained from a commercial source (Charles River Germany, Sulzfeld, Germany).

Phenotypic Analysis. H&E sections of neoplastic tissues were examined using established diagnostic criteria for neoplasia of the rat endocrine system (7, 8). Immunohistochemical staining of thyroid C cells was performed after antigen retrieval using a commercial antihuman Calcitonin antibody (Signet Laboratories, Dedham, MA). Deparaffinized 5-μm sections were boiled for 20 min in 0.01 M citrate buffer [8 mM sodium citrate dihydrate, 2 mM citric acid, (pH 6.0)] in a microwave oven followed by a 15 min incubation in PBS containing 10% serum at 37°C. Primary antibody was applied at a dilution of 1:500 and incubated for 1 h at 25°C. Labeling was visualized with an immunoperoxidase-coupled second antibody according to standard procedures. Negative controls were performed by omitting the primary antibody. Periodic acid-Schiff-stained sections of lens tissue were prepared as described previously (9) and examined by a pathologist experienced in ocular pathology.

Analysis of the Inheritance Pattern. Transmission of the phenotype was initially determined in two cohorts of F1 hybrid animals that were obtained by mating SD^{we} animals with either wild-type Sprague Dawley or Wistar rats. Final confirmation of the recessive mode of inheritance was made using populations of F2 intercross and backcross animals. Intercross animals were obtained by crossing ($SD^{we} \times$ Wistar) F1 hybrid rats, whereas backcross animals were generated by mating SD^{we} mutant animals with ($SD^{we} \times$ Wistar) F1 hybrids.

Molecular Genetic Analysis. The integrity of the *Menin* and *Ret* genes was examined by direct sequencing of reverse transcription-PCR amplification products obtained from adrenal tissues of affected Sd^{we} rats, their nonaffected littermates, and wild-type Sprague Dawley rats (all of the primer sequences are available from the corresponding author electronically). PCR and sequencing primers for rat *Menin* cDNA were designed using the published sequence (Ref. 10; GenBank accession no. AB023400). The rat *Ret* cDNA sequence was determined from reverse transcription-PCR products generated using PCR primers based on mouse-human gene homologies and the published rat gene sequences (GenBank accession no. AJ298999-AJ299017). The 5′ untranslated region of the rat *Ret* cDNA was sequenced from PCR products generated by 5′ rapid amplification of cDNA ends amplification. Sequence alignment analysis was performed using MacVector 6.0 (Genetics Computer Group, Madison, WI).

Statistical Analysis. Statistical analysis was performed using the StatView 4.5 software package (Abacus Concepts, Berkeley, CA). Life expectancy was plotted using the Kaplan Meier statistic and significance determined using the Log-Rank (Mantel-Cox) test. Analysis of the frequency of the transmission of the mutant phenotype in the intercross and backcross populations was made using Fischer's exact test. Values are presented as mean ± SD.

RESULTS

Cataract Formation. Affected animals develop macroscopically visible bilateral cataracts in the first few weeks of life. Histological analysis of the eyes at 5–7 months demonstrated a mature cataract (Fig. 1)

[4] A. Fritz, unpublished observations.

Fig. 1. The phenotypic features characterizing the SD^{we} phenotype. *A,* nodular C-cell hyperplasia. The expansion of the C-cell population between isolated thyroid follicles is revealed by immunohistochemical staining (*brown* reaction) for calcitonin. *B,* pheochromocytoma showing uniform neoplastic cells and infiltration of the adipose tissue (*arrow*). *C,* paraganglioma arising near to the aorta abdominalis (*arrow*) in the retroperitoneum. *D,* cataract. The lens has lost its biconvex shape; the lens substance shows degenerative lens fibers with clefts and vacuoles. The *yellow* center of the lens represents a nuclear cataract.
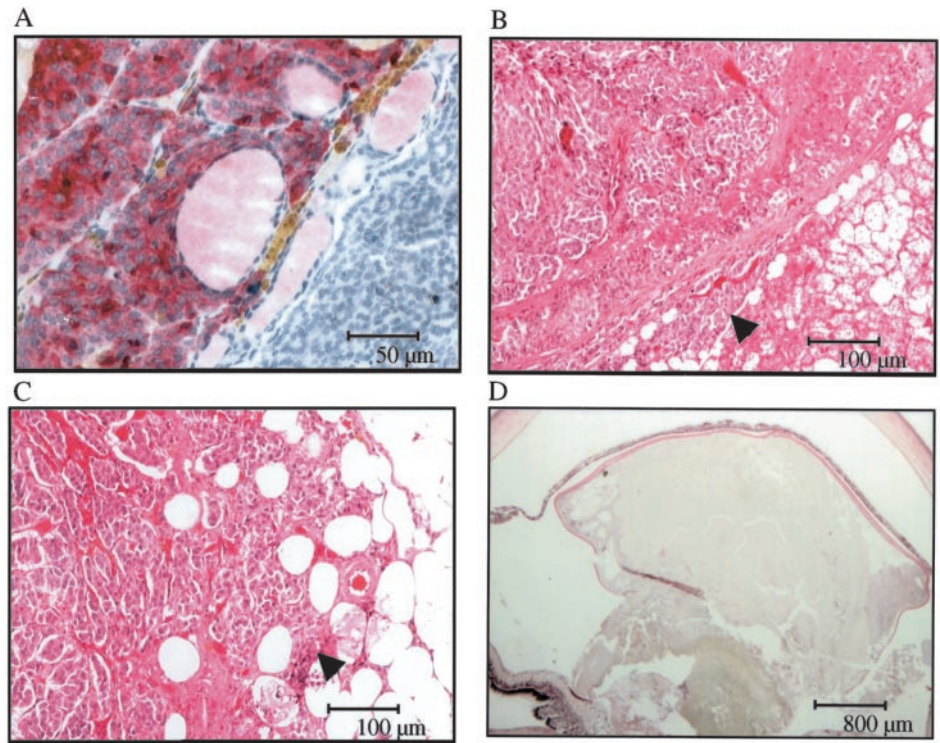
with a clear cornea, a well-differentiated retina, and an intact anterior uvea. Alterations typical of nuclear cataract are a swollen lens epithelium, rupture of the posterior lens capsule, formation of deep clefts and vacuoles in the lens cortex, the presence of a yellow, amorphous structure in the lens core, and Morgagnian vesicles. No evidence of an inflammatory reaction was seen. Differentiation of the secondary lens fiber cells was also disturbed, because cell nuclei in the lens bow region are disarranged. Cataracts were absent in only 1 of 191 animals that subsequently developed MENs. Indeed, these two clinical features did not segregate during long-term breeding or in the genetic crosses detailed below, making it very probable that both arise through a single gene mutation. None of the >100 mutations causing cataracts in the mouse have been associated with neuroendocrine malignancy to date.

Neuroendocrine Neoplasia. Although the mutant phenotype was initially identified by the presence of cataracts, it was subsequently found to include neoplasia of multiple endocrine tissues (Fig. 1). In order of frequency the most commonly observed neoplasms in affected (cataract-bearing) animals were pheochromocytoma, paraganglioma (extra-adrenal pheochromocytoma), thyroid medullary C-cell hyperplasia/neoplasia, adenoma of the anterior pituitary gland, and hyperplasia of the parathyroid gland (Table 1). The neoplasias affecting adrenal, parathyroid, and medullary thyroid tissues were almost exclusively bilateral. Paragangliomas were always multiple and usu-

Table 1 *Comparison of human MEN phenotypes with the neoplastic tissue involvement in SD^{we} rats*

Phenotype	MEN1	MEN2A	MEN2B	SD^{wea}
Pituitary adenoma	+^{b}			77%
Insulinoma	+			None
Parathyroid adenoma	+	+		65%
Phaeochromocytoma		+	+	95%
Medullary thyroid hyperplasia		+	+	78%
Paraganglioma		+		85%
Neuroma			+	None

^{a} The frequency of each neoplasia in SD^{we} rats was determined at the time of death and includes only macroscopically visible tumors. In this part of the study SD^{we} rats were drawn from the breeding colony on the basis of the presence of cataracts.
^{b} + denotes occurrence of this tumor type in each disease entity.

ally located in close proximity to the aorta and large arteries. Animal welfare considerations require sacrifice of animals as soon as symptoms appear; therefore we believe that the greater frequency of pheochromocytoma in these animals reflects the greater morbidity associated with this tumor. The prompt sacrifice of animals also prevents us from determining the absolute incidence rates for each malignancy and from following tumor progression.

The phenotypic pattern of endocrine malignancy in these animals most closely resembles that seen in the human form of MEN2A, although some important differences exist (Table 1), notably the presence of pituitary adenoma and cataracts (Fig. 2).

Inheritance of the Phenotype Is Autosomally Recessive. The mode of inheritance was examined by selective breeding studies. Crosses between pairs of affected animals were largely nonproductive, whereas the strain could be readily maintained by mating affected animals with nonaffected littermates. No sex linkage was detected for the phenotype; in a pool of 191 animals developing cataracts and MEN-like symptoms the ratio of males:females was 1.03, indicating that the mutation is autosomal. The first indication that the phenotype was attributable to a recessive gene mutation was the complete lack of phenotype in F1 hybrid animals. None of the 23 (SD^{we} × SD wild-type) F1 or 55 (SD^{we} × Wistar wild-type) F1 animals developed cataracts or the MEN-like neoplastic phenotype. The F1 animals had an average life span of 551 ± 79 days, similar to published data for the Sprague Dawley strain (11).

The recessive mode of inheritance was confirmed by the reappearance of the complete phenotype in both F2 intercross and F2 backcross cohorts. In the F2 intercross between (Sd^{we} × Wistar) F1 rats 24 of 123 (19.5%) of the offspring developed cataracts with MEN, which is not significantly different from the frequency predicted for a recessive trait (25%; $P = 0.39$). The F2 backcross between parental SD^{we} animals and (SD^{we} × Wistar) F1 hybrids yielded 31 of 66 (47%) offspring with cataract and MEN-like malignancies, compared with the expected frequency for a recessive trait of 50% ($P = 0.84$; not significant).
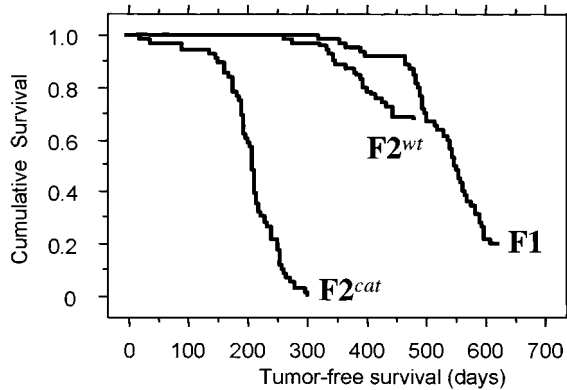
Fig. 2. Kaplan-Meier analysis of tumor-free survival of F1 and F2 cohorts. Data for the F2 backcross and F2 intercross animals are pooled (see "Results" for details). F2^{cat} denotes animals developing cataract, all of which subsequently developed MEN-like neuroendocrine neoplasia. F2^{wt} denotes animals that were phenotypically normal. None of the F2^{wt} or F1 animals developed MEN-like disease.

Lifetime studies in F2 animals were performed on cohorts initially segregated at 1 month of age by the presence of cataracts (F2cat and F2wt populations, see Fig. 2) revealed that animals with cataracts all went on to develop the MEN phenotype and exhibited a dramatically shorter life span, averaging 243 ± 60 days in F2cat animals compared with 519 ± 63 days in their F2wt nonaffected littermates (Fig. 2; Log-Rank $\chi^2 = 161.47$; P < 0.0001). The life spans of nonaffected F2 animals and F1 animals were not significantly different.

The results from all three of the genetic crosses are consistent with a recessive inheritance pattern. Therefore, we can conclude that affected animals (SD^{we}) are homozygous for the mutant allele whereas nonaffected littermates are heterozygous for the mutant allele.

Molecular Genetics. We examined the cDNA sequences of the *Menin* and *Ret* genes in somatic tissues obtained from homozygous mutants, heterozygous littermates, and wild-type Sprague Dawley rats. The *Menin* cDNA sequence in all three of the inferred Sprague Dawley genotypes was identical, with all showing a single difference to the published sequence: a silent G/A substitution at nucleotide 1671. Because this sequence was found in both affected and nonaffected animals we assume it represents a sequence polymorphism between the Sprague Dawley rat strain used in our study and the Wistar strain sequenced in the original GenBank submission.

No sequence differences in the coding region of the *Ret* gene were identified between mutant and nonmutant (heterozygote and wild-type) animals. Two single base differences (silent polymorphisms) were identified between the Sprague Dawley sequence and the GenBank deposit (strain not given). We again assume that these sequence differences are sequence polymorphisms, because they are present in both mutant and nonmutant animals.

DISCUSSION

We report the characterization of a recessive germ-line mutation responsible for the development of a MEN-like syndrome in the rat. Affected animals show the unique association of bilateral cataracts with bilateral adrenal pheochromocytoma, multiple extra-adrenal pheochromocytoma, bilateral medullary thyroid cell neoplasia, bilateral parathyroid hyperplasia, and pituitary adenoma. All of the phenotypic features develop within the first year of life, and average survival of affected animals is only 243 days (maximum survival 354 days).

Whereas pituitary adenomas are commonly encountered in aged rats of many strains, including the Sprague Dawley strain used here, our animals typically develop pituitary tumors within the first year of life. A number of other endocrine malignancies infrequently develop

in inbred rats, but these sporadic tumors do not commonly affect multiple tissues or involve multiple tumors, and do not as a rule appear in young animals. For example, Long-Evans strain rats develop pituitary adenoma and pheochromocytoma. However, these tumors only affect aged animals, do not involve other endocrine tissues, and have not been shown to be the result of a recessive germ-line mutation (12).

The recessive mode of inheritance of the trait, and the presence of cataracts, is in clear contrast to all three human forms of hereditary MEN, which are transmitted in a dominant manner through the germ-line (1–4). The spectrum of endocrine tissues affected is also somewhat different to those encountered in any of the human MEN syndromes. Whereas the involvement of parathyroid, chromaffin, and C cells is similar to that seen in MEN2, the presence of pituitary adenomas is typically restricted to MEN1. A similar mixed-type multiple neuroendocrine neoplasia has been reported in mice deficient for one copy of the *Rb1* gene (13) or with combined deletions of the cyclin dependent kinase inhibitors p18 and p27 (14). A true MEN1 phenotype has been described in *Men1* heterozygous knockout mice (15). These animals develop a dominantly acting MEN1-like phenotype in association with pituitary neoplasia but lack paraganglioma formation and cataracts seen in our animals. The "mixed" phenotype MEN may be specific to laboratory rodents but more probably may be because of a common pathway of neuroendocrine neoplasia.

The role of the cell cycle control proteins in MEN-like disease suggests the phenotype we observe may be because of deregulated cycle control in neuroendocrine tissues. The absence of angioma, renal carcinoma, and hemangioblastoma excludes the reported allelic variants of the von Hippel-Lindau syndrome gene *Vhl* (16), whereas the lack of neurofibromatous tumors excludes the neurofibromatosis gene *Nf1* (17). The phenotype is also quite distinct from the rare forms of familial endocrine neoplasias involving pheochromocytoma or abdominal paragangliomas (18, 19). Because no evidence could be found for mutation of either the *Menin* and *Ret* genes that are associated with human forms of MEN we conclude that the mutant phenotype is the result of a novel gene mutation.

The closeness of the phenotype to the human forms of MEN2 suggests that the mutated gene may be involved in a regulatory pathway linked to RET, possibly at a cell cycle regulatory point in RET action. However, the recessive mode of inheritance suggests a loss-of-function mutation, which is in contrast to the dominantly acting gain-of-function *RET* mutations seen in human MEN2. A loss-of-function mutation of the type typically encountered in tumor suppressor genes would also appear to be an unlikely scenario, especially as heterozygous animals remain neuroendocrine tumor free. Inactivation of RET itself is also unlikely, as homozygous deletion in mice generates a non-MEN phenotype with intestinal agangliosis, renal agenesis, and a defect in the superior cervical ganglia (20). An alternative explanation is a gene dosage effect, with inactivation of a regulatory component leading to modest activation of the RET signal pathway. In heterozygous animals this would not be sufficient to induce the phenotype but in the homozygous state could elicit an overproliferation of endocrine tissues. In this model the hypothetical mutation could affect the RET signal pathway through loss of a repressor, gain of function of an activator, or by alteration in transcription frequency of a regulatory gene.

Identification of the lesion may shed light on the mechanism of action of MEN genes in humans and may assist in developing an appropriate therapeutic strategy for endocrine neoplasias. The availability of an animal model will also permit the study of the effect of modifying gene loci, which may play an important role in determining the severity of the MEN phenotype in humans.

ACKNOWLEDGMENTS

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