Allelic Loss at the Tuberous Sclerosis (*Tsc2*) Gene Locus in Spontaneous Uterine Leiomyosarcomas and Pituitary Adenomas in the Eker Rat Model

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Hereditary renal carcinomas (RCs) develop in virtually all Eker rats by the age of one year. Investigation of extra-renal primary tumors co-occurring in Eker rats late in life (at 2 years) additionally revealed enhanced development of hemangiosarcomas of the spleen, uterine leiomyosarcomas and pituitary adenomas, although the demonstrated predilection for these extra-renal tumors was not as complete as with RCs. We identified the germline mutated tuberous sclerosis (*Tsc2*) gene as the predisposing Eker gene and revealed the tumor suppressor nature of *Tsc2* gene function in renal carcinogenesis. In the present study, we examined allelic loss at the *Tsc2* gene locus in uterine leiomyosarcomas and pituitary adenomas developing in hybrid F1 rats carrying the Eker mutation as well as in pituitary adenomas from non-carrier rats. We detected loss of heterozygosity in 4 of 11 uterine leiomyosarcomas (36%) and 11 of 31 pituitary adenomas (35%) from Eker rats but in none of 9 pituitary adenomas from non-carrier rats ($P < 0.05$), suggesting that inactivation of the *Tsc2* gene is also a critical event in the pathogenesis of these extra-renal tumors. Our present data indicate that there might be different pathways for tumorigenesis of pituitary adenomas between Eker and non-carrier rats.

Key words: Hereditary cancer — Uterine leiomyosarcoma — Pituitary adenoma — Tuberous sclerosis (*Tsc2*) gene — Loss of heterozygosity

The development of hereditary renal carcinomas (RCs) in the rat, originally reported by R. Eker in 1954, is an example of a Mendelian dominantly inherited predisposition to a specific cancer in an experimental animal.[1] Recently, we have established a new conserved linkage group on rat 10q and human 16p13.3, whereby the Eker mutation was found to be tightly linked to the tuberous sclerosis (*Tsc2*) gene,[2] and finally identified a germline mutation in the *Tsc2* gene itself.[3, 4] The heterozygous Eker rat typically develops RCs through multiple stages from early preneoplastic lesions (e.g., phenotypically altered tubules) to adenomas by the age of one year.[5] Loss of heterozygosity (LOH) was found for rat chromosome 10 markers in RCs[6, 7] and even in the earliest preneoplastic lesions, e.g., phenotypically altered tubules[8] developing in hybrid F1 rats carrying the Eker mutation, supporting the theory of a second somatic mutation (second hit) as a rate-limiting step of renal carcinogenesis in the Eker rat model and a tumor suppressor nature for *Tsc2* gene function.

Investigation of extra-renal primary tumors occurring in Eker rats late in life additionally revealed probable hemangiosarcomas of the spleen, probable leiomyosarcomas of the uterus,[9, 10] and pituitary adenomas,[10] al-though the observed predilection for extra-renal tumors was not as complete as with RCs.[10] Thus, the Eker rat provides an opportunity for understanding organ/cell-type specific carcinogenesis.

Although aging non-carrier rats also have been observed to have pituitary adenomas, an increase in number and earlier onset of pituitary adenomas was observed in Eker rats.[10] Our questions are, whether a second somatic *Tsc2* gene mutation (second hit) also occurs in extra-renal tumors in Eker rats, and whether the same genetic changes are found in pituitary adenomas of non-carrier rats.

MATERIALS AND METHODS

Animals Founder rats (two males and three females) carrying the Eker mutation were kindly provided by A. G. Knudson (Fox Chase Cancer Center). Eker rats have been bred on a Long Evans (LE) background (Charles River Breeding Laboratory) by brother × sister mating and maintained pathogen-free in the Animal Facility of the Cancer Institute since 1991. All animals were housed and treated in accordance with institutional guidelines. Eker rats were diagnosed as carriers by detecting microscopic kidney tumors following unilateral nephrectomy around 3–6 months of age, as well as the germline mutation in the *Tsc2* gene.[3]

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Experimental procedure Two male rats carrying the Eker mutation (Eker/+, LE strain of Charles River Breeding Laboratory) were mated with several normal female rats (+/+) of the LE strain (Kiwa Breeding Laboratory) and the inbred Brown Norway (BN) strain (Charles River Japan, Inc.) to produce hybrid F1 rats showing DNA polymorphisms.[7] Rats at two years of age were killed to check tumor incidences macro- and microscopically.[10]

DNA samples We analyzed 11 uterine and 31 pituitary tumors from Eker rats (Eker/+), and 9 pituitary tumors from non-carrier rats (+/+). Fresh samples of tumors and normal liver were obtained, and immediately frozen at −80°C. High-molecular-weight genomic DNAs were extracted by digestion with 2% sodium dodecyl sulfate (SDS)/proteinase K followed by extraction with phenol.[11]

Histology The remaining parts of the excised tumors were examined microscopically for histology. Tissues were fixed in 10% formalin and a routine histological examination was carried out on hematoxylin and eosin (HE)-stained paraffin sections. For determining the cell origin of uterine tumors, the following monoclonal antibodies were used for immunohistochemical studies[12]: anti-alpha-smooth muscle actin (SMA, DAKO), anti-actin (Actin, Amersham), anti-muscle actin HHF35 (HHF35, ENZO), anti-S-100 protein (S100, DAKO), anti-desmin (DS, Bio-Science), and anti-vimentin (VM, DAKO). For assessing pituitary tumor functions, two monoclonal antibodies, anti-adrenocorticotrophic hormone (ACTH, DAKO) and anti-prolactin (PL, DAKO), were used for immunohistochemical studies and Grimelius' staining was performed.

Southern-blot analysis DNAs (10 μg per lane) were digested with restriction enzymes revealing polymorphisms between LE (USA) and LE (Japan)/BN strains, electrophoresed on 1% agarose gels, and transferred onto nylon membranes (Biodyne, Pall Biosupport) in 0.4 N NaOH. As previously reported,[7] the blots were hybridized overnight in a solution containing 0.2 M NaHPO₄ (pH 7.2), 1 mM EDTA (pH 8.0), 1% bovine serum albumin and 7% SDS, with ^{32}P-labeled DNA probes (1–3×10⁶ cpm/ml) prepared by a random oligonucleotide-priming procedure using a Hybriroter (Model HR-1, Nippon Genetics). The filters were washed twice in 1× SSC, 0.1% SDS at room temperature for 15 min each, and 65°C for 30 min. They were exposed to X-ray films with an intensifying screen at −70°C for 1–2 days.

Polymorphic DNA probes One partial cDNA (2.3 kb) of rat Tsc2 gene was isolated from the cDNA library of an adult rat kidney using a cDNA fragment of the human counterpart as a probe (ref. 3 and T. Kobayashi et al., manuscript in preparation). The mouse IRF-1 gene (gift of Dr. T. Taniguchi, Tokyo University) and the mouse protamin-1 (PRM-1) gene (gift of Dr. R. Yeung, Fox Chase Cancer Center; originating from the American Type Culture Collection) were used as polymorphic DNA markers of rat chromosome 10 as previously reported.[13]

PCR The oligonucleotide primers of rat Tsc2 locus (5′-TGTGGCCTAACCTTTGAGGT-3′ and 5′-CTGTGT-TAGAACGTGGGAGA-3′) were made using a DNA synthesizer (Milligen/Biosearch, Division of Millipore). Polymerase chain reaction (PCR) was performed in 10 μl of reaction mixture containing 100 ng of the genomic DNA, 1 pmol/μl of the primers, 1 mM MgCl₂, 200 μM each of dGTP, dATP, dTTP, and dCTP, and 0.5 unit of Tth DNA polymerase (Biotech). Thirty-five cycles of amplification, each consisting of denaturation for 60 s at 92°C, annealing for 60 s at 55°C, and extension for 90 s at 72°C, were performed with a thermal programmer (QTII or QTPI; Nippon Genetics). The products of the PCR were digested with the RsaI restriction enzyme, and were electrophoresed on 3% Nusieve agarose gels (FMC Bioproducts) and visualized with ethidium bromide. The oligonucleotide primers of rat interleukin-3 (IL3) (5′-CTGCTTAGAGCCTTCACACA-3′ and 5′-AGGAAT-TCGTCCAGGTTTACT-3′) were used for PCR as previously reported.[7, 8, 13]

Definition of LOH When 50% reduction in signal intensity was detected, it was judged as LOH, as reported previously.[7]

RESULTS AND DISCUSSION

In the uterine tumors, small foci of atypical cells were observed histologically surrounded by abundant collagen fibers (Fig. 1a). Tumor cells had large nucleoli and were rich in chromatin, showing bizarre shapes. The nucleo-cytoplasmic ratio was high. They were positive for Actin, HHF35, SMA (Fig. 1b), VM, and DS, but negative for S100 immunohistochemically, suggesting that they might be of smooth muscle origin, e.g., leiomyomas/leiomyosarcomas rather than stromal sarcomas.[10]

Histologically, the pituitary tumors in Eker rats were all adenoma, and not essentially different from tumors in non-carriers.[10] Immunohistochemical stainings showed that pituitary tumor cells were negative for ACTH and PL, suggesting that they might be non-functioning tumors, and some tumor cells were positive for Grimelius' staining (data not shown).

As shown in Fig. 2, LOH on chromosome 10 was clearly detected in uterine and pituitary tumors, and the wild-type allele was always lost, consistent with the two-hit hypothesis, as with RCs.[7] We detected LOH in 4 of 11 uterine tumors (36%) and 11 of 31 pituitary tumors (35%) developing in Eker rats as summarized in Fig. 3. None of 5 hemangiosarcomas of the spleen exhibited
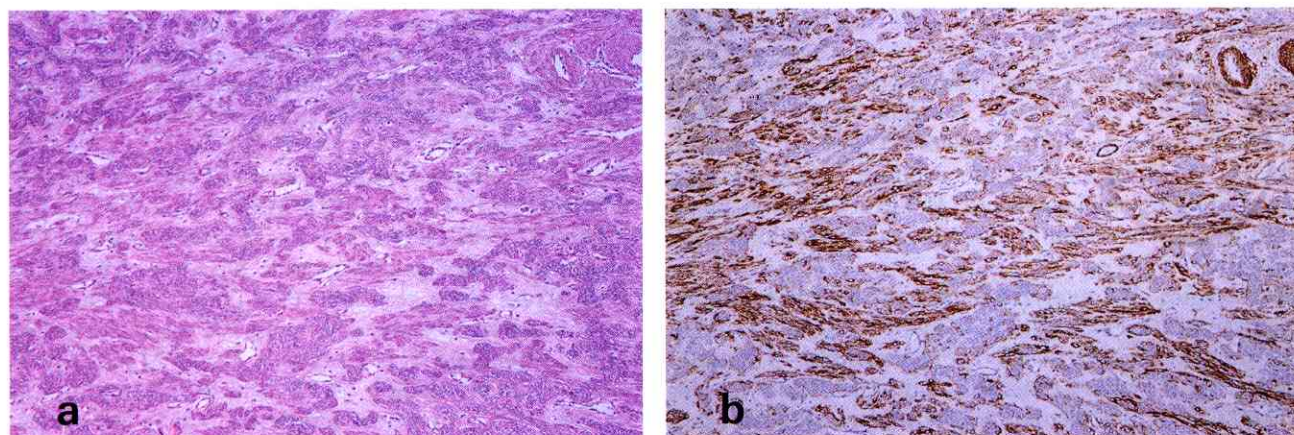
Fig. 1. a: The tumor is composed of swirling bands of smooth muscle cells mixed with varying amounts of collagen (HE, ×50). b: The cytoplasma of tumor cells show a positive reaction (α SMA, ×50).
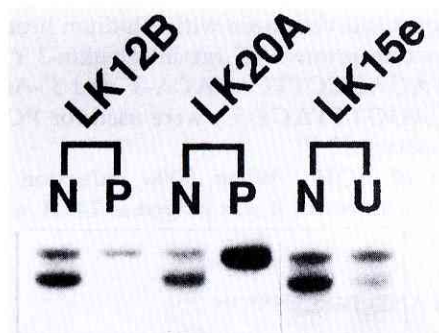

Fig. 2. Representative Southern blot analysis (*Eco*RI digestion) of pituitary and uterine tumors with the 3′ half of rat *Tsc2* cDNA as a probe. N, P, and U indicate samples of normal liver, pituitary tumors, and uterine tumors, respectively. *LK12B, LK20A,* and *LK15e* are hybrid F1 rats [LE (USA) and LE (Japan)] carrying the Eker mutation. Although in uterine tumors the normal cellular component seems to exist to some degree, loss of the wild-type allele originating from the LE (Japan) strain was shown for these tumors.

Fig. 3. Summary of patterns of LOH in LOH-positive samples. Numbers below indicate the number of samples that belong to each LOH pattern.

LOH (data not shown). One explanation might be contamination with appreciable normal cellular components in such tumors and/or the small number of cases investigated. Out of a total of 15 positive cases of uterine leiomyosarcomas and pituitary adenomas, LOH could be detected with a 2.3 kb partial cDNA clone of rat *Tsc2* in all but one. In only a single pituitary adenoma was this not the case, although this tumor had LOH at the IRF-1 and IL3 loci. This 2.3 kb cDNA clone is located in the 3′ half of the *Tsc2* gene (5.5 kb), but in Southern blot analysis with the 5′ half (3.3 kb) of the cDNA as a probe, rearranged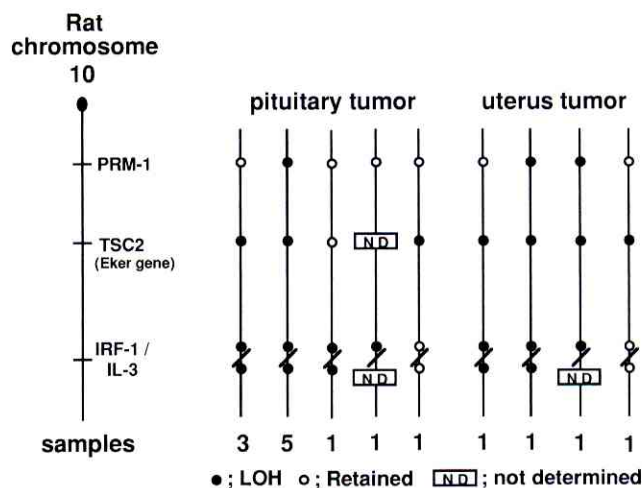 DNA bands were observed in the exceptional case, as shown in Fig. 4. We confirmed that DNA rearrangement occurred within the 5′ end of the *Tsc2* gene (data not shown). Out of a total of 15 cases that had LOH, one uterine leiomyosarcoma and one pituitary adenoma had LOH at only the *Tsc2* gene locus. It was therefore concluded that they had smaller deletions of the wild-type allele than the others. Previously we reported that LOH could be detected in 6 of 10 (60%) RCs originating in Eker rats.[7] The frequencies of detected LOH in uterine leiomyosarcomas and pituitary adenomas were thus a little lower (but not statistically significantly) than for their RCs counterparts. We now plan to
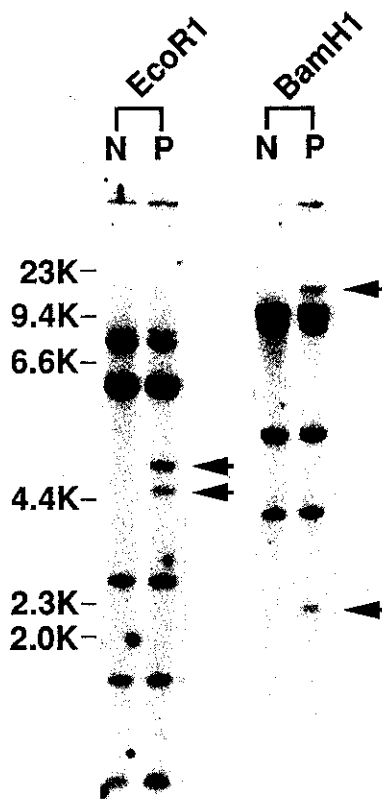
Fig. 4. Representative Southern blot analysis of a pituitary tumor with the 5′ half of rat *Tsc2* cDNA as a probe. The pituitary adenoma (P) has rearrangement bands (arrow) with both *Eco*RI and *Bam*HI digestion.
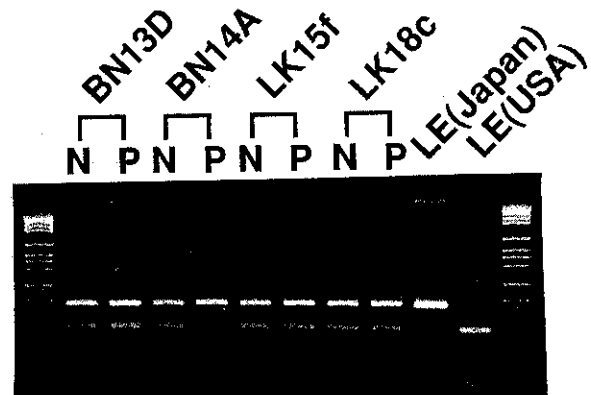

Fig. 5. Digestion with the *Rsa*I restriction enzyme after PCR with oligonucleotide primers of the *Tsc2* gene. *BN13D* and *BN14A* are hybrid F1 rats carrying the Eker mutation, while *LK15f* and *LK18c* are normal hybrid F1 rats [LE (USA) and LE (Japan)]. LE (Japan) and LE (USA) indicate normal controls for the LE (Japan) and LE (USA) strains, respectively. In *BN14A*, loss of the wild-type allele originating from the BN strain was clearly shown in the pituitary adenoma. However, LOH could not be detected in non-carrier rats. We checked 2 other LOH-positive cases and got the same pattern of *BN14A* (P) (data not shown).

search for small mutations (e.g., point mutations or small deletions) in cases with no LOH.

Aging non-carrier rats also had pituitary adenomas at a frequency of around 20%.[10] Although all 9 rats were hybrid F1 rats [LE (USA) and LE (Japan)], conventional Southern blot analyses using total *Tsc2* cDNA clone as the probe were not informative in these non-carrier rats. However, fortunately, we found a polymorphism between CTG/LE (USA) and CTA/LE (Japan) in an exon, which corresponds to codon 1249 CTG (Leu) of human *Tsc2* cDNA,[3, 14] and clearly detected heterozygosity by digesting PCR products with the *Rsa*I restriction enzyme, as shown in Fig. 5. The difference between the mutation rates of 11 of 31 pituitary adenomas (35%) in Eker rats and none of 9 pituitary adenomas (0%) proved to be statistically significant ($P < 0.05$). Histologically, there were no differences between pituitary adenomas of Eker rats and those of non-carrier rats,[10] but this does not preclude different pathways for tumorigenesis. Similar observations were earlier reported for

von Hippel-Lindau disease (VHL)-related pheochromocytomas and sporadic pheochromocytomas.[15]

In human sporadic pituitary tumors, allelic loss at chromosome 11 has been identified, as well as in those associated with multiple endocrine neoplasia type 1 (MEN1).[16] Although transgenic mice containing a disrupted retinoblastoma tumor suppressor gene (RB) often develop pituitary tumors, these tumors originate from the intermediate, and not the anterior, lobe of the pituitary gland,[17] and LOH could not be detected in human pituitary tumors at the RB gene locus.[18] This is clearly different from our case.

In human uterine leiomyomas, cytogenetic abnormalities of chromosome 6p, 12q and 14q were reported,[19] although there is no syntenic relationship between these human chromosomes and rat chromosome 10.[2] Recently, Walker *et al.* described a non-random abnormality of rat chromosome 4 in leiomyosarcoma cell lines from Eker rats.[20] However, to our knowledge, the present data provide the first demonstration of genetic changes in early tumorigenesis of uterine leiomyosarcomas.

The Eker rat bears a single gene mutation with a dominant predisposition and develops tumors in four different organs — the kidney, spleen, uterus and pituitary. It thus offers us a novel animal model of cancer predisposition syndromes. However, the extra-renal tumors do not appear to demonstrate as complete a penetrance as RCs, and the phenotype of tuberous sclerosis

in humans differs from that of the Eker rat, except for the occurrence of RCs (in human, angiomyolipomas are more common). At present, we do not have a good explanation for the variation in phenotypic manifestations, but future analysis of the Eker rat model should provide insights into phenotype-specific mutations, species-specific differences in tumorigenesis and organ/cell-type specific carcinogenesis.

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