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Advances in Brief

Early Detection of Knudson's Two-Hits in Preneoplastic Renal Cells of the Eker Rat Model by the Laser Microdissection Procedure[1]

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Abstract

Hereditary renal cell carcinomas invariably develop by the age of 1 year in Eker rats. At the histological level, renal cell carcinomas develop through multiple stages from early preneoplastic lesions (*e.g.*, phenotypically altered tubules) to adenomas. We previously reported that ionizing radiation induces additional tumors (large adenomas and carcinomas) in a linear dose-response relationship and that loss of heterozygosity (LOH) at chromosome 10, where the predisposing tuberous sclerosis (*Tsc2*) gene is localized, was found in the renal cell carcinomas which developed from hybrid F1 rats carrying the Eker mutation, indicating that in heterozygotes two events (one inherited, one somatic) are necessary to produce at least large adenomas and carcinomas. This study was designed to examine LOH in the earliest preneoplastic lesions, using a laser microdissection procedure. We could accurately dissect single altered renal tubules out of freeze-dried sections and clearly detected LOH in 4 of 19 altered tubules (21%). This is the first demonstration of LOH in single renal tubules. Our present results support the theory of a second, somatic mutation (second hit) as rate-limiting step of renal carcinogenesis in the Eker rat model of dominantly inherited cancer and the tumor suppressor nature of the *Tsc2* gene function.

Introduction

RC[3] 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, the predisposing gene of the Eker rat was mapped to the proximal part of rat chromosome 10 (2, 3). 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 (4) and finally identified a germline mutation in the *Tsc2* gene (5, 6).

We previously reported that: (*a*) according to our radiation experiment, renal tumors in heterozygotes appear to involve two hits, one of which is inherited in the Eker rat and the other of which can be induced by a somatic event (7); (*b*) LOH was found for rat chromosome 10 markers in the RCs developing in hybrid F1 rats carrying the Eker mutation, supporting a recessive, tumor suppressor nature for the predisposing gene (2, 8). Our question is whether a second, somatic mutation (second hit) already occurs in the earliest preneoplastic lesions, *e.g.*, phenotypically altered renal tubules (9), in the Eker rats. In this study, we microdissected the altered tubules from freeze-dried sections using a laser beam (10) and effectively amplified DNA by the PCR method. We clearly detected LOH of the wild type allele in them at the predisposing gene locus. The present results might further strengthen the "two-hit" hypothesis in carcinogenesis.

Materials and Methods

Animals. Founder rats carrying the Eker mutation were kindly provided by Dr. Alfred G. Knudson (Fox Chase Cancer Center, Philadelphia, PA) in 1991. Eker rats were bred on a Long Evans (LE) background (Charles River Breeding Laboratory) by brother × sister mating and maintained pathogen free in the Animal Facility of 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 at 3–6 months of age. Three carrier rats were sacrificed to obtain kidneys that contain early preneoplastic lesions. Of three rats, two were hybrid F1 rats [RC/+, LE(USA)/inbred brown Norway (BN) strain (Charles River Japan, Inc.), 112 and 142 weeks] and the other one was a hybrid F2 rat (RC/+, 10 weeks) which was produced by a hybrid male F1 rat [RC/+, LE(USA)/BN] and a normal female rat (+/+) of the LE strain (Kiwa Breeding Laboratory, Japan). Excised kidneys were cut sagittally in two, frozen with a sheet of paper on dry ice, and stored at −80°C.

Preparation of Sections and Histology. Eighteen-μm serial sections were prepared with a cryostat microtome. As shown in Fig. 1, they were divided into groups every three serial sections. The second section of each succeeding group was stained with PAS for histological examination. The first and third sections of each set of three serial sections, which were used for microdissection of lesions, were freeze-dried at −40°C and stored under vacuum at −45°C until use.

Microdissection. Phenotypically altered renal tubules (Fig. 2), which are the earliest lesions histologically, were microdissected out of freeze-dried sections with a micropreparative laser device (BTG Biotechnik GmbH, Munich, Germany) equipped with a liquid dye laser, a microscope with a TV camera, and a computer-assisted digitizer system with regard to PAS-stained sections as reported previously (10). We analyzed 19 altered tubules. The microdissected tissue samples were placed in Eppendorf tubes and stored at −45°C until use for PCR. After the microdissection procedure, we compared each freeze-dried section with the PAS-stained section which corresponded to it and confirmed that the lesion could be dissected.

PCR. The oligonucleotide primers of rat interleukin 3 (5′-CTGCTTAGAGCCTTCACACA-3′ and 5′-AGGAATTCGTCCAGGTTTACT-3′) (3) were made with a DNA synthesizer (Milligen/Biosearch, Division of Millipore). The interleukin 3 gene is located on rat chromosome 10 and 26 cM apart from the predisposing gene (3, 4). Microdissected samples were used for PCR without prior isolation of DNA (11). Each sample was suspended in 25 μl of a reaction mixture containing 1 pmol/μl of the primers, 0.75 mM $MgCl_2$, and 200 μM concentrations each of dGTP, dATP, dTTP, and dCTP. The mixture was heated at 94°C for 10 min, and the PCR was started by adding 2 units of Taq polymerase (Biotech). Thirty-five cycles of amplification, each consisting of denaturation for 60 s at 94°C, annealing for 60 s at 55°C, and extension for 90 s at 72°C, were performed with a thermal programmer (Perkin Elmer; Takara Biomedicals). The products of the PCR were electrophoresed on 1.5% low melting point agarose gels (Gibco BRL) and visualized with ethidium bromide (8).

Results and Discussion

We could clearly detect LOH on chromosome 10 even in the earliest preneoplastic lesions (*e.g.*, phenotypically altered tubules) as

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[3] The abbreviations used are: RC, renal cell carcinoma; LOH, loss of heterozygosity; PAS, periodic acid-Schiff.

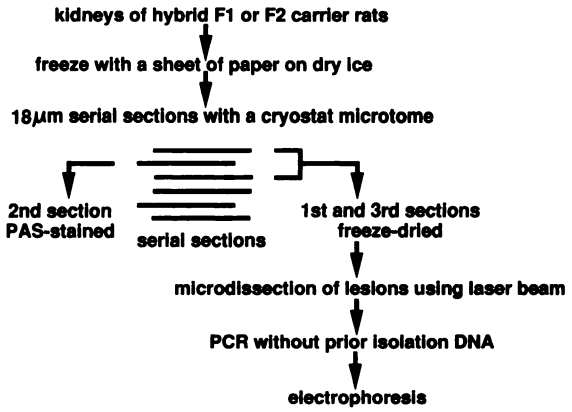

Fig. 1. Microdissection approach.

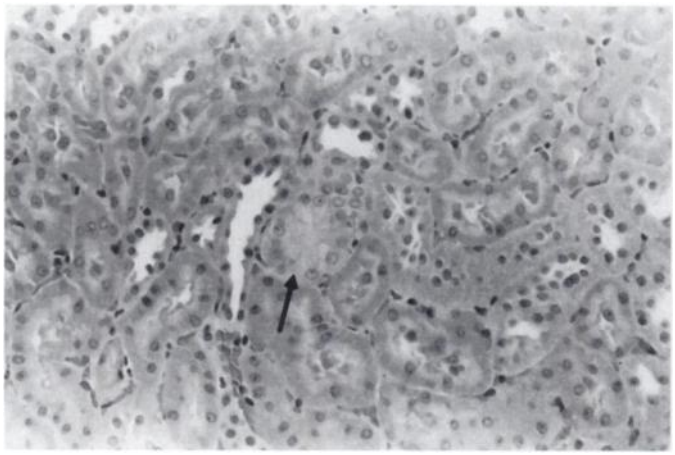

Fig. 2. The earliest preneoplastic lesion (*arrow*) dissected in this study. × 100.

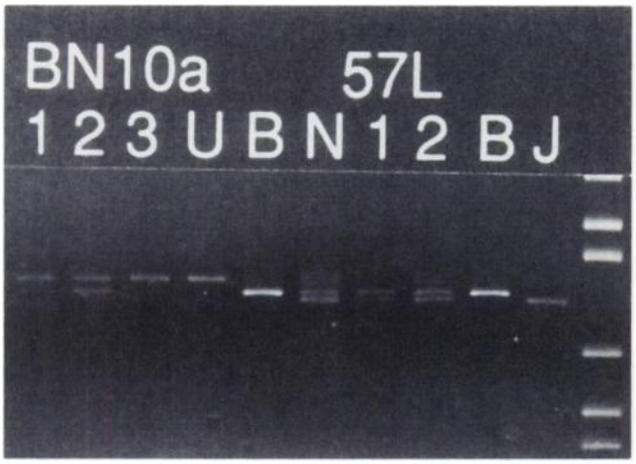

Fig. 3. PCR with oligonucleotide primers of interleukin 3. *BN10a* and *57L* are the animals. *Numbers*, individual preneoplastic tubules; *N*, normal renal tubule; *U, B, J*, normal controls of LE(USA), brown Norway, and LE(Japan) strains, respectively. *Right ordinate*, size markers.

shown in Fig. 3. The wild type allele was always lost, which was consistent with the two-hit hypothesis.

At the histological level, phenotypically altered tubules appear 2 months after birth in carrier rats (7, 12, 13). We previously reported that renal carcinomas had LOH at chromosome 10 (8), but it was unclear when LOH occurred. In this study, we examined LOH in the earliest microscopic precursor lesions, using a laser microdissection approach. By this approach, we could dissect tiny lesions that were too small to be taken with ordinary procedures, and LOH could be clearly detected in them. We think that this microdissection approach is useful in the analysis of molecular events in tiny lesions that are 100 μm in diameter.

We could detect LOH in 4 of 19 altered tubules (21%), even if the marker DNA used in the study is located about 26 cM from the predisposing *Tsc2* gene (3, 4). We can expect an increase in the rate of LOH with predisposing *Tsc2* gene itself, although in the present study a PCR primer of the *Tsc2* gene locus showing polymorphism was not available. We previously reported that in spontaneous RCs, 60% (6 of 10) showed LOH covering over 30 cM and, in contrast, 0% (0 of 9) in ethylnitrosourea-induced RCs (14) using several DNA markers located on rat chromosome 10 (8). Now we can characterize the second hit (intragenic mutations including point mutation) in LOH-negative lesions at the DNA-sequencing level of the predisposing *Tsc2* gene.

Although human tuberous sclerosis is known to be associated with development of RCs, nothing is known to date about the molecular mechanism. Thus, the Eker rat continues to be a valuable experimental model for studying *Tsc2* gene function and its role in renal carcinogenesis.

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