Identification of a Locus for Susceptibility to Renal Cell Carcinoma in the Long-Evans Cinnamon Rat

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ABSTRACT. The Long-Evans Cinnamon (LEC) mutant rat shows higher incidence of renal cell carcinomas induced by a treatment with the chemical carcinogen *N*-diethylnitrosamine, as compared to the normal control rat. We performed the first genome-wide scan for genes responsible for susceptibility to chemically induced renal cell carcinoma in an F₂ intercross obtained by mating the LEC and Fischer-344 (F344) rats. The genotype of 71 (F344 × LEC) F₂ progenies was determined with the use of 338 simple sequence length polymorphisms (SSLPs) spread over the genome. The F₂ rats which carried renal cell carcinoma were shown to possess the incidence of homozygosity of the LEC allele which is higher than that of the other genotypes at SSLP markers on chromosome 5 ($\chi^2 = 17.5$ for *D5Rat21*). Our linkage analysis has led to the revelation of a novel gene that influences susceptibility to renal cell carcinoma on rat chromosome 5.—**KEY WORDS:** LEC rat, linkage, renal cell carcinoma.

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Renal cell carcinoma is the most common malignancy of the adult kidney. Environmental factors, such as cigarette smoking and chemical carcinogen exposure, are likely to be involved in the development of renal cell carcinoma. Additionally, genetic predisposition could constitute additional, unknown risk factors. In human, genetic factors characterized by highly penetrant alleles represent important risk factors for some rare tumors [10]. It is very difficult, however, to assess the possible contribution of genetic predisposing factors in the pathogenesis of tumors that do not give rise to familiar clustering [16, 17], such as renal cell carcinoma other than renal tumors observed in von Hippel-Lindau disease [23]. Experimental animal models, in which genetic susceptibility and resistance to renal cell carcinoma exist, do provide an opportunity for genetic investigation.

The Long-Evans Cinnamon (LEC) rat was established as a new mutant strain that spontaneously develops necrotizing hepatic injury and hepatocarcinoma [18, 28]. Thereafter, it was reported that the LEC rat shows excessive hepatic copper accumulation, which is thought to lead to the development of the hepatic injury and subsequent hepatocarcinogenesis possibly due to copper cytotoxicity [12, 13]. We have shown that the causative gene for abnormal copper metabolism in this mutant is the rat homologue of the Wilson's disease gene, designated as *ATP7B*, which encodes copper-transporting P-type adenosine triphosphatase responsible for intracellular copper delivery, and thus that this rat is an ideal animal model for Wilson's disease [15, 26].

On the other hand, it was reported that spontaneous development of renal cell carcinoma is observed in long-surviving LEC rats [6]. We found that the LEC rat is more susceptible to renal cell carcinoma induced by an injection of the carcinogen *N*-diethylnitrosamine, as compared to the normal control rat. Here, we report on the first genome-wide scan for genes responsible for susceptibility to chemically induced renal cell carcinoma in the LEC rat. The results show that a novel gene on chromosome 5 is essential for the onset of renal cell carcinoma.

Inbred strains of LEC and Fischer-344 (F344) rats are bred under specific pathogen-free conditions in the Institute for Animal Experimentation, University of Tokushima School of Medicine, which is coded as Tj (Tokushima, Japan). F344/Tj rat was used as a control. The (F344 × LEC) F₂ population of 71 rats was produced by crossing F344 females with LEC males to produce F₁ rats, and then intercrossing F₁ × F₁ to produce F₂. All rats had free access to tap water and standard laboratory chow and were maintained at a 12-hr light and dark cycle (6 am/6 pm).

The F₂ rats were phenotyped for renal cell carcinoma as follows. Rats received subcutaneous injections of 100 mg/kg body weight *N*-diethylnitrosamine (Tokyo Chemical Industry Co., Tokyo, Japan) three times at 6, 8 and 10 weeks of age, and then were sacrificed at the age of 46 weeks. Mortality from treatment to the end of the experiment was 0%. The kidney tumors were checked macroscopically. Thereafter, the kidney tissues including tumors were sliced, fixed in 10% buffered formalin (pH 7.2) and embedded in paraffin. Deparaffinized sections were stained with hematoxylin and eosin, and screened for the presence of renal cell carcinoma. All the gross kidney tumors were identified as renal cell carcinoma microscopically. Spleen was dissected and frozen at -80°C for subsequent isolation of high molecular weight DNA. This study conforms to the

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guidelines for the care and use of laboratory animals of University of Tokushima School of Medicine.

We purchased primer pairs for over 800 simple sequence length polymorphism (SSLP) markers [4, 7] (Research Genetics, Inc., Huntsville, AL), and chose a set of 338 markers which were polymorphic in LEC and F344 rats. We genotyped 71 (F344 female $\times$ LEC male) F_2 progeny and the parental strains by PCR amplification of the polymorphic markers. PCR was performed using 200 μ l microtubes and a PC-800 thermal cycler (ASTEC Co., Ltd., Fukuoka, Japan). The reaction volume was 10 μ l, containing final concentrations of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100 and 200 μ M each of dATP, dCTP, dGTP and dTTP. Genomic DNA (50 ng), 0.5 U of *Taq* polymerase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 0.66 μ M of each primer were used. The PCR was started at 94°C for 2 min and continued for 40 cycles of 1 min at 94°C, 1.5 min at 55°C and 1.5 min at 72°C, followed by additional 72°C for 10 min. PCR products were mixed with the loading buffer supplemented with xylene cyanol and bromophenol blue dyes in 25% glycerin, and subjected to electrophoresis on 4% MetaPhor™ (FMC BioProducts, Rockland, ME) agarose gel which contained 1 mg/ml ethidium bromide for photographing the PCR products under UV-light using Polaroid film type 667 (Polaroid, Cambridge, MA). Some PCR products were fluorescence-labeled and analyzed by electrophoresis in standard denaturing sequencing gel using an ABI Prism 377 gene scan system (Perkin Elmer, Co., Emeryville, CA).

Genetic markers were mapped relative to each other with the MAPMAKER/EXP computer package [11]. A candidate for laboratory typing error was identified by an error detection procedure on the MAPMAKER/EXP computer package, which is a systematic method based on incorporating the possibility of error into the usual likelihood model for linkage analysis [14]. Potential errors were rechecked against photographs and, where necessary, the genotyping was repeated. Linkage of markers to renal cell

carcinoma was evaluated by χ^2 tests in 3×2 contingency tables (StatView, SAS Institute, Inc., Cary, NC). Linkage was defined by a χ^2 exceeding 13.8 [24].

At the age of 46 weeks, after treatment with *N*-diethylnitrosamine three times at 6, 8 and 10 weeks of age, approximately 14% of the LEC rat carried microscopically visible renal cell carcinomas, whereas the carcinomas were detected in ~5% of the F344 rats (Table 1). This indicates that the LEC rat shows higher incidence of chemically induced renal cell carcinoma by *N*-diethylnitrosamine than the normal control rat. This higher incidence in the LEC rat was confirmed in other experiments (data not shown). To investigate the genetic control of the susceptibility to renal cell carcinoma in the LEC rat, we generated 71 (F344 $\times$ LEC) F_2 intercross. Eight rats (11%) of the animals in the intercross carried renal cell carcinoma (Table 1). Four of 39 male F_2 and 4 of 32 female F_2 developed renal cell carcinoma, indicating no significant sex-related difference (Table 1). In an attempt to detect renal cell carcinoma susceptibility genes, we genotyped 338 polymorphic SSLP markers throughout the rat genome on the F_2 intercross. The markers were on average 4.7 cM apart, and approximately 90% of the genome was within 10 cM of an informative marker. We detected evidence of linkage to renal cell carcinoma on chromosome 5 markers (Table 2, Fig. 1). The highest χ^2 was obtained for *D5Rat21* ($\chi^2 = 17.5$, $P=0.0002$). We found the bias to homozygotes for the LEC allele at the chromosome 5 markers in the F_2 rats with renal cell carcinoma (Table 2). Therefore, our linkage study

Table 1. Susceptibility to renal cell carcinoma

Strain	No. of rats tested		No. of rats with tumors	
	Male	Female	Male	Female
LEC	21	—	3	—
F344	21	—	1	—
(F344 $\times$ LEC) F_2	39	32	4	4

—, No data.

Table 2. Associations of markers within chromosome 5 region around *D5Rat21* with renal cell carcinoma in the (F344 $\times$ LEC) F_2 intercross

Locus name	Distance	No. of rats with tumors			No. of rats without tumors			χ^2	<i>P</i> value
		ff	fl	ll	ff	fl	ll		
	cM								
<i>D5Mit3</i>	10.3	1	3	4	25	31	7	8.6	0.0158
<i>D5Mit4</i>	0.8	1	2	5	16	41	6	15.3	0.0005
<i>D5Rat21</i>	1.4	1	2	5	18	40	5	17.5	0.0002
<i>D5Wox3</i>	8.2	1	2	5	16	40	7	13.9	0.0010
<i>D5Mgh23</i>	—	0	4	4	15	38	10	6.2	0.0445

The number of rats that are F344 homozygote (ff), F344/LEC heterozygote (fl) and LEC homozygote (ll) for SSLP marker are listed. Linkage of markers to renal cell carcinoma was evaluated by χ^2 tests in 3×2 contingency tables. Distances between loci are described in centiMorgans, calculated with MAPMAKER/EXP program.

Chromosome 5

Fig. 1. Linkage map of SSLP markers on chromosome 5. Distances are given in centimorgans calculated by use of Kosambi map function. Markers on the same line are those that can not fit into a small interval and are arranged next to the upper marker flanking the interval they are located in along with their distance from that marker. *On the markers, evidence of linkage to renal cell carcinoma was detected ($\chi^2 > 13.8$).

demonstrated that the genome region around *D5Rat21* on chromosome 5 contains a novel gene that influences susceptibility to renal cell carcinoma possibly in a recessive manner. We should note that the linkages of markers in this genome region with renal cell carcinoma were confirmed by Kruskal-Wallis test ($P=0.0005$ for *D5Mit4*, $P=0.0002$ for *D5Rat21*, and $P=0.001$ for *D5Wox3*).

Our data show that a susceptibility gene to renal cell

carcinoma maps to the genome region near *D5Rat21* on rat chromosome 5. At the chromosome 5 susceptibility gene, LEC alleles act possibly in a recessive mode of inheritance to enhance the susceptibility to renal cell carcinoma. These results will be confirmed using a backcross obtained by mating ($F_{344} \times \text{LEC}$) F_1 and LEC rat. Half (50%) of the F_2 progenies homozygous for the LEC allele at the susceptibility gene carried renal cell carcinoma, whereas approximately 14% of the LEC rat did. The higher penetrance of the susceptibility gene in the F_2 progenies suggests existence of a modifier gene that is derived from the F344 rat and enhance the susceptibility gene effect on renal cell carcinoma development.

It has been reported that the LEC rat manifests hepatocellular carcinoma [18, 28]. Although genes involved in the hepatocellular carcinoma remain unclear, hepatic injury caused by copper accumulation seems to promote hepatocarcinogenesis presumably due to continuous compensatory cell proliferation, caused by cell injury and the resulting loss of cells, which may enhance the frequency of DNA damage and/or the fixation of DNA mutation [19, 21]. The LEC rat possesses three times higher copper content in the kidney than the F344 rat [6]. Our linkage analysis demonstrated that the *Atp7b* gene mutation on chromosome 16, which leads to copper accumulation in the liver and kidney of the LEC rat [15, 26], shows no linkage with renal cell carcinoma (data not shown). This suggests that copper accumulation in the LEC kidney caused by the *Atp7b* gene mutation is not responsible for the onset of renal cell carcinoma. However, this could not deny the possibility that the copper accumulation in the kidney leads to the promotion of renal carcinogenesis through enlarging the size of renal cell carcinoma.

The mouse and human genomic regions homologous to the chromosome 5 genome region around *D5Rat21* would be 4C3-C7 for mouse and 1p32-p31 and 9p23-p21 for human, respectively [2, 25]. These regions of the rat, mouse or human genomes include v-jun avian sarcoma virus 17 oncogene homolog (*Jun*) locus [1], cyclin-dependent kinase inhibitor 2A (*Cdkn2a*) locus [8], cyclin-dependent kinase inhibitor 2B (*Cdkn2b*) locus [9], and superoxide dismutase 2 (*Sod2*) locus [3], as physiologically relevant, positional candidate genes. The oncogene *Jun* is the putative transforming gene of avian sarcoma virus 17; it appears to be derived from a gene of the chicken genome and has homologs in several other vertebrate species [1]. *Jun* gene encodes a nuclear protein with enhancer binding properties of AP1, and a chromosomal region containing this gene is involved in both translocations and deletions in human malignancies [5]. The *Cdkn2a* gene was originally symbolized as *Mts1* (for multiple tumor suppressor 1). Chromosomal region containing this gene, that encodes a previously identified inhibitor (p16) of cyclin-dependent kinase 4 (CDK4), is involved in chromosomal inversions, translocations, heterozygous deletions, and homozygous deletions in a variety of malignant cell lines [8]. The p16 protein binds to CDK4 and inhibits the ability of CDK4 to

interact with cyclin D and stimulate passage through the G1 phase of the cell cycle [20]. The CDKN2B is another member of the family for the inhibitor of CDK4 and CDK6 which both promote cell cycle progression through G1 the phase [9]. The *Cdkn2b* gene is located adjacent to *Cdkn2a* and is co-deleted in a high proportion of established human cancer cell lines [9]. The *Sod2* gene encodes an intramitochondrial free radical scavenging Mn-enzyme that is the first line of defense against superoxide produced as a toxic by-product of oxidative phosphorylation [3]. In this terms, it should be noted that the Mn-SOD levels in the LEC rat are lower than those of the normal control, especially in the liver and kidney [22], and that the amount of 8-hydroxydeoxyguanosine in DNA, a marker of active oxygen-derived DNA damage, is increased in the liver and kidney of the LEC rat [27]. These informations will help us to apply a positional candidate gene approach to clarify whether these genes are involved in the pathogenesis of renal cell carcinoma in the LEC rat.

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