Mapping a Sex Hormone–Sensitive Gene Determining Female Resistance to Liver Carcinogenesis in a Congenic F344.BN-*Hcs4* Rat

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Abstract

Hepatocellular carcinoma (HCC) is prevalent in human and rodent males. Hepatocarcinogenesis is controlled by various genes in susceptible F344 and resistant Brown Norway (BN) rats. *B* alleles at *Hcs4* locus, on RNO16, control neoplastic nodule volume. We constructed the F344.BN-*Hcs4* recombinant congenic strain (RCS) by introgressing a 4.41-cM portion of *Hcs4* from BN strain in an isogenic F344 background. Preneoplastic and neoplastic lesions were induced by the "resistant hepatocyte" protocol. Eight weeks after initiation, lesion volume and positivity for proliferating cell nuclear antigen (PCNA) were much higher in lesions of F344 than BN rats of both sexes. These variables were lower in females than in males. Lesion volume and PCNA values of male RCS were similar to those of F344 rats, but in females corresponded to those of BN females. Carcinomatous nodules and HCC developed at 32 and 60 weeks, respectively, in male F344 and congenics and, rarely, in F344 females. BN and congenic females developed only eosinophilic/clear cells nodules. Gonadectomy of congenic males, followed by β-estradiol administration, caused a decrease in *Ar* expression, an increase in *Er-α* expression, and development of preneoplastic lesions comparable to those from BN females. Administration of testosterone to gonadectomized females led to *Ar* increase and development of preneoplastic lesions as in F344 males. This indicates a role of homozygous *B* alleles at *Hcs4* in the determination of phenotypic patterns of female RCS and presence at *Hcs4* locus of a high penetrance gene(s), activated by estrogens and inhibited/unaffected by testosterone, conferring resistance to females in which the *B* alleles provide higher resistance. (Cancer Res 2006; 66(21): 10384-90)

Introduction

Epidemiologic evidence suggests the existence in humans of a genetic predisposition to hepatocellular carcinoma (HCC) involving low-penetrance genetic variants (1). Due to human genetic heterogeneity, detection of genetic variants in a population is difficult. Rodent strains with different susceptibility to HCC allowed mapping of various susceptibility and resistance loci controlling growth and differentiation of neoplastic lesions (1).

HCC is prevalently male associated in humans and rodents (2–5). Endocrine ablation studies showed enhancement of hepatocarcinogenesis by testosterone and inhibition by ovarian hormones (4–6). These effects depend on functional specific receptors (7, 8). The antioxidant property of estrogens (9) was proposed to explain their inhibition on tumor development, but other reports indicate increased production of mitochondrial superoxide in rat hepatocytes and HepG2 cells following estrogen treatment (10) and a positive correlation between estradiol/testosterone ratio and malonyldiadehyde levels in rats exposed to ethanol (11). Because sex hormones regulate growth hormone secretion, it was hypothesized (12) that growth hormone mediates sex difference in susceptibility to HCC. Growth hormone deficiency in mice carrying a point mutation in the gene encoding the growth hormone releasing hormone receptor ($Ghrhr^{lit}$) suppresses hepatocarcinogenesis of both sexes. However, manipulation giving a feminized pattern of growth hormone secretion to rat males leads to higher basal serum levels of this hormone than in normal males (13).

Previous studies (14) allowed mapping of *H*epato*c*arcinogenesis in *f*emale 1 and 2 (*Hcf1* and *Hcf2*) loci conferring to BR mice resistance to hepatocarcinogenesis inhibition by ovarian hormones. However, the genes responsible for resistance of females to hepatocarcinogenesis remain unknown. Sex dimorphism of the susceptibility to hepatocarcinogenesis has never been studied in rats genetically resistant to hepatocarcinogenesis and the role of *H*epato*c*arcinogenesis *s*usceptibility (*Hcs*) genes has not been evaluated. Seven *Hcs* loci, determining the susceptibility to HCC, have been identified in the rat genome (1). Among them, allelic variant of *Hcs4* locus in the Brown Norway (BN) rat strain, on centromeric RNO16, plays a role in the control of neoplastic liver nodule volume (15). To investigate whether this locus is implicated in the determination of the genetic trait of rats, we constructed a recombinant congenic strain (RCS) by introgressing a portion of *Hcs4* of BN strain in an isogenic Fisher 344 (F344) background. RCS showed a sex-differentiated phenotype compatible with the presence of a potent hormone-sensitive gene at *Hcs4*, responsible for the resistance to hepatocarcinogenesis of female rats.

Materials and Methods

Animals and treatments. F344 and BN rats (140-160 g; Charles River-Italia, Calco, Italy) were fed and housed as reported (16). RCS was generated by marker-assisted selection (ref. 17; Fig. 1) with three backcrosses of (BN × F344)F1 males with F344 females to produce *FB* at *Hcs4* locus with as much as *FF* homozygous elsewhere. Rats *FB* at *Hcs4* were intercrossed

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-06-2881

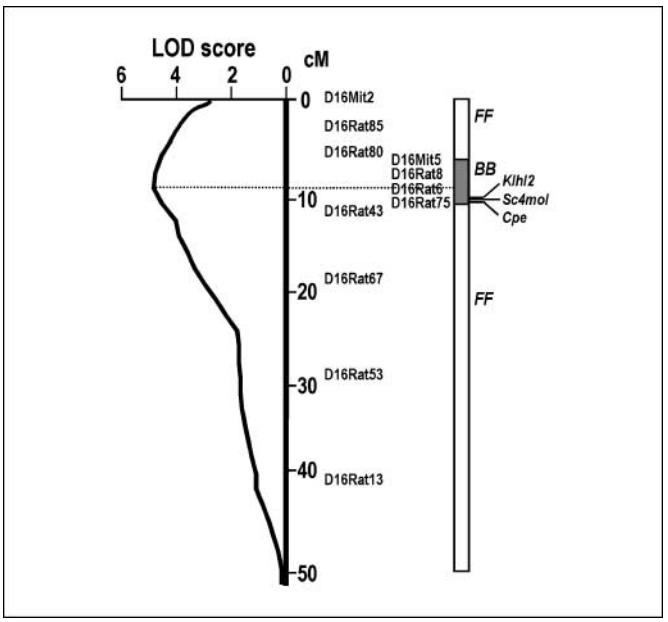

Figure 1. Genetic map, quantitative trait loci scan, and congenic line interval (*gray vertical column*) defining the *Hcs4* locus on rat chromosome 16. F344 male was mated with BN female rats and BFF1 females were backcrossed to F344 males. Rats carrying segments of the ~25-cM region, corresponding to *Hcs4*, from BN rats, were used for two additional backcrosses. Brother and sister siblings, heterozygous at BN-derived *Hcs4* segment and prevalently homozygous at F344-derived remaining genome, were intercrossed. The N1 rats homozygous for a restricted portion BN-derived *Hcs4* segment, including LOD score peak, and with increased *FF* homozygosity at the remaining genome, were selected for intercrossing until the N9 generation. In every generation, siblings with the progressive restriction of the *BB* homozygous *Hcs4* and increasing *FF* homozygosity at the remaining genome were selected for subsequent intercrossing. *Horizontal dotted line,* LOD score peak at 9.04 cM from the centromere. The http://ratmap.gen.gu.se and http://www-genome.wi.mit.edu; http://www.jax.org databases were used to locate the microsatellite markers along the genome. The http://www.ncbi.nlm.nih.gov/entrez/ database was used to identify the genes mapping in the congenic segment of *Hcs4* locus. Data relative to unrestricted *Hcs4* in (BN × F344)F2 rats were taken from ref. 15.

until N9 generation. N9 animals were the founders of the RCS F344.BN-*Hcs4* line. Male and female congenics (120-150 g) were gonadectomized by excision of ovaries and testes and epididymis, respectively, under anesthesia. When indicated, gonadectomized males and females were treated 24 hours after surgery with β-estradiol 17-acetate and testosterone acetate (Sigma-Aldrich Srl, Milano, Italy), respectively, with a daily dose of 3.3 mg/kg i.p. for 3 weeks, followed by two doses weekly of 10 mg/kg i.p. for 5 weeks.

Rats were divided into 10 groups, consisting of male and female F344, BN, F344.BN-*Hcs4* rats, gonadectomized congenics, and gonadectomized rats plus antagonistic hormone. Rats were initiated by diethylnitrosamine and treated according to the "resistant hepatocyte" model (18). Gonadectomized rats ± antagonistic hormone were subjected to carcinogens 2 weeks after surgery. Fifteen rats of each group were killed 8 weeks after initiation by bleeding through the thoracic aorta under anesthesia. Ten male and 10 female rats of F344, BN, and F344.BN-*Hcs4* strains were killed 32 and 60 weeks after initiation. Controls were represented by five rats per group, not treated with carcinogens. Livers were excised and processed for H&E staining, glutathione-*S*-transferase 7-7 (GST 7-7), and proliferating cell nuclear antigen (PCNA) immunohistochemistry (16), and PCNA index was expressed as the percentage of PCNA(+) nuclei. Number per cubic centimeter, mean volume of lesions, volume fraction, and percentage of remodeling foci of altered hepatocytes and early nodules (larger than a liver lobule, compressing surrounding liver) were evaluated by morphometric analysis (15). The lesions were considered remodeling when at least 20% of their surface was negative to GST 7-7 immunostaining. Animals received humane care, and study protocols were in compliance with the guidelines of our institution for the use of laboratory animals.

Genotyping. Genomic DNA was purified from rat tails and genotyping was done as described (15) using 128 polymorphic microsatellite markers (Supplementary Table S1; Roche Diagnostic S.p.A., Monza, Italy) distributed throughout all autosomes and X chromosome (one marker/9.53 cM, except for chromosome 16, where genotyping was done particularly densely).

Reverse transcription-PCR. Quantitative reverse transcription-PCR (RT-PCR) was done with 75 to 300 ng of cDNA using an ABI Prism 7000 and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Primers for *Cpe, Sc4mol, Er-α, Er-β, Ar*, and *RNR-18* were chosen by the Assays-on-DemandTM Products (Applied Biosystems). Cycling conditions were 10 minutes at 95°C, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute (16). Due to the lack of specific probes for quantitative RT-PCR of *Kelch-like-2*, its expression was evaluated by semiquantitative comparative RT-PCR (19) with the following specific primers: *Kelch-like-2* (forward/reverse): 5′-ATGGCTCACTGAGAGTTCGCA/5′-TGATAAACCTG-TGCTGCCGTC; *RNR-18*; 5′-GGCCCGAAGCGTTTACTTTGAA/5′-GCATCGC-CAGTCGGCATCGTTTAT. Cycling variables were 30 minutes at 55°C, 2 minutes at 95°C, 1 minute at 55°C, and 1 minute at 72°C, for 30 cycles in a GeneAmp PCR system 9700 (Applied Biosystems).

Assays. To determine γ-glutamyl transpeptidase, liver homogenates in 50 mmol/L Tris-Cl/0.5% Triton X-100 (pH 9.5) were incubated at 25°C in a reaction mixture containing 4 mmol/L L-γ-glutamyl-*p*-nitroanilide, 50 mmol/L glycylglycine, 50 mmol/L Tris-Cl, 10 mmol/L $MgCl_2$ (pH 8.2). Data are expressed as absorbance increase at 405 nm/min/g liver. Testosterone and β-estradiol were determined by Immunoassay (Abbott Diagnostic Laboratories, Abbott Park, Chicago, IL) following the protocol of the manufacturer.

Statistical analysis. Data are expressed as mean ± SD. Comparisons between means were made by Student's *t* test and Tuckey-Kramer test. $P < 0.05$ was considered significant.

Results

General findings. No variation among rat groups of both sexes occurred for diet consumption and body weight (not shown). Liver weights per 100-g body weight of F344, BN, and congenic rats not treated with carcinogens ranged from 2.98 to 3.5 g in males and from 3.22 to 3.36 g in females. Eight weeks after initiation, liver weights increased by 52% and 35% in male F344 and congenic rats, respectively, and did not change in male BN rats and females of all strains. This probably reflected differences in lesion development linked to strain and sex. Liver weight decreased by 22% in male gonadectomized RCS but recovered after treatment with β-estradiol. Gonadectomy did not modify liver weight of female RCS treated or untreated with testosterone. Thirty-two weeks after initiation, liver weight of RCS increased by 64% to 70% and 18% to 22% in carcinogen-treated males and females, respectively.

Generation of F344.BN-*Hcs4* RCS. *Hcs4* locus, located on the centromeric side of RNO16 (Fig. 1), controls the size of preneoplastic and neoplastic liver lesions in F344 and BN rats (15). We constructed a RCS (Fig. 1) by the selective introgression, in an isogenic *FF* background, of a 4.41-cM segment of this locus from BN rats, between D16Mit5 and D16Rat75, including the LOD score peak, previously located at 9.04 cM (15). A detailed genotypic analysis of N9 RCS (Supplementary Table S1) showed the uniform presence of the *B* allele in the 4.41-cM segment of *Hcs4* locus, with *F* alleles present in the remainder RNO16, except a few rats heterozygous and one *BB* homozygous at D16Rat53. Additional *BB* homozygous and *FB* heterozygous sites were randomly distributed in other chromosomes. The percentage of *B* alleles at the 4.41-cM segment of *Hcs4* locus, scanned with microsatellites at an average distance of 1.1 cM, represented 3.16% of the entire

genome in both sexes. Randomly distributed *B* alleles outside RNO16 represented 9% of genome.

Effect of BN-Hcs4 on hepatocarcinogenesis. Eight weeks after initiation, lesion number was 2.5-fold higher and size 13.8-fold lower in male BN than F344 rats (Fig. 2). In male F344.BN-*Hcs4* rats, lesion number was intermediate but not significantly different from that of F344 and BN rats, and the volume did not significantly differ from the F344 value. Volume fraction reflected the situation of lesion number and volume, indicating similar rates of lesion development in male congenic and F344 strains. In females of F344 and BN rats, lesion number was higher and volume and volume fraction were lower than in males, with male/female ratio of ~4/1.5-fold for volume/volume fraction in both strains. Female F344.BN-*Hcs4*, different from male rats, behaved as the parental BN strain, characterized by very low volume and volume fraction values. Increase in lesion number, associated with volume decrease, probably depended on reduction in lesion confluence and/or coalition (Supplementary Fig. S1). Indeed, the levels of γ-glutamyl transpeptidase activity, another marker of preneoplastic liver, were in good agreement with that of the volume fraction, excluding artifactual results of morphometric analysis. Differences in lesion volume between strains and sexes were paralleled by analogous changes in PCNA index of liver lesions (Fig. 2), indicating a sharp influence of the genetic background on growth rate of initiated hepatocytes. The percentage of remodeling lesions was higher in BN than in F344 and congenic rats of both sexes. Remodeling was slightly but significantly higher in females than males of F344 rats.

Thirty-two weeks after initiation, 38.6 ± 12.06 nodules per liver, with mean diameter of 4.19 ± 1.3 mm, developed in male congenics, 70% of which were well-differentiated or moderately differentiated HCC, whereas no carcinomas developed in females, in which numerous clear/eosinophilic cell nodules occurred. Carcinomatous nodules were also present in liver of male F344 rats, but only in 1 of 10 female rats. Only eosinophilic/clear cell nodules developed in liver of BN rats (Supplementary Fig. S2). Sixty weeks after initiation, all male F344 and congenics and 80% of BN rats developed well-differentiated and moderately differentiated HCC. Liver nodules, present in 100% of female rats of all strains, showed histologic patterns of HCC in only 3 of 10 female F344 rats. In liver of other female F344 rats and all female BN and congenic rats, only eosinophilic/clear cell nodules were detected (Supplementary Fig. S2).

Effect of sex steroid hormones. These observations suggest a genetic control of the sex-differentiated susceptibility to rat liver cancer. To verify this hypothesis, we evaluated interstrain differences in the expression of androgen receptor (Ar) and estrogen receptors α and β, as well as the effect of sex hormones on the expression of these receptors and the development of preneoplastic liver in the F344.BN-*Hcs4* strain. No significant differences occurred in testosterone and β-estradiol plasma levels of F344, BN, and congenic strains of both sexes (data not shown). Comparative analysis of *Ar* and *Er-α* expression (Fig. 3, *left*) showed lower values of both receptors in normal liver of BN than F344 male rats, whereas the expression levels of congenic rats were comparable to those of F344 rats. A similar pattern was found for *Er-α* expression

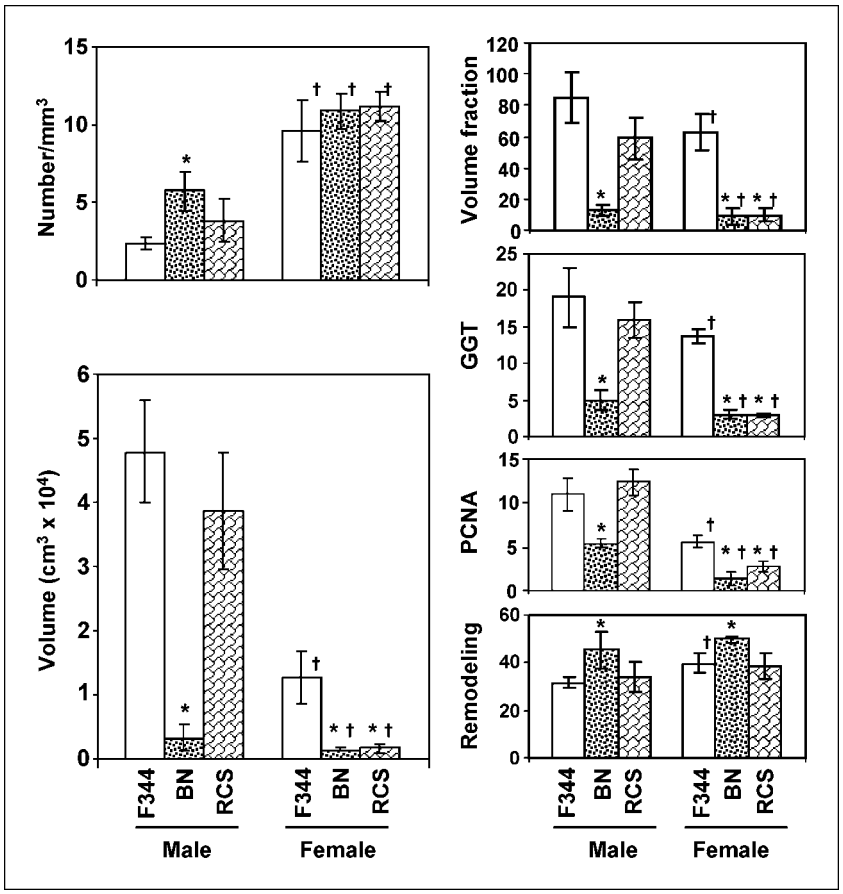

Figure 2. Number, volume, volume fraction, PCNA index, and remodeling of GST 7-7–positive lesions, and γ-glutamyl transpeptidase activity per gram of liver of F344, BN, and F344.BN-*Hcs4* rats 8 weeks after initiation. Liver lesions were induced by the "resistant hepatocyte" model in rats initiated by diethylnitrosamine. Two thousand to 4,000 GST 7-7(+) hepatocytes per liver were counted to determine PCNA index. PCNA index is expressed as the percentage of positive nuclei in liver lesions, and γ-glutamyl transpeptidase (*GGT*) as enzymatic activity per gram of liver. Number, volume, and volume fraction of liver lesions were calculated by computer-assisted analysis of GST 7-7(+) lesions. Remodeling represents the percentage of lesions exhibiting irregular margins and nonuniform pattern of at least 20% of GST 7-7 immunostaining. *Columns,* mean of 5 to 13 rats; *bars,* SD. *, $P < 0.001$, different from F344. †, $P < 0.01$, different from male rats.

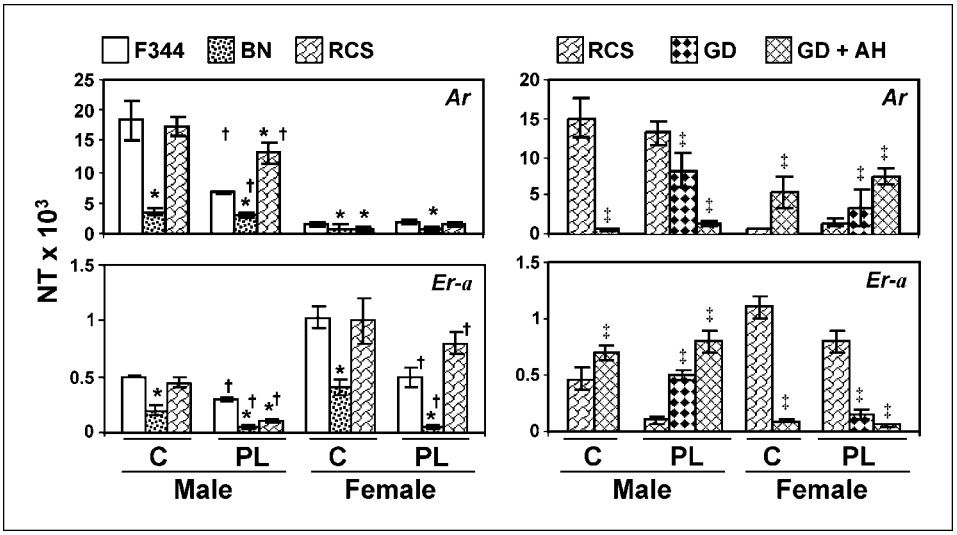

Figure 3. Expression of *Ar* and *Er-α* genes in normal and preneoplastic liver of F344, BN, and congenic rats. When indicated, congenic rats were gonadectomized and treated with antagonistic hormones as described in Materials and Methods. Data of quantitative RT-PCR. *Columns,* mean of *N* target (NT) of five to nine rats; *bars,* SD. NT = $2-\Delta Ct$, where ΔCt value of the sample was calculated by subtracting the average Ct value of the target gene from the average Ct value of the *RNR-18* gene. *C,* control (normal liver); *PL,* preneoplastic liver; *GD,* gonadectomy; *AH,* antagonistic hormones. *, $P < 0.05$, different from F344. †, $P < 0.01$, different from normal liver. ‡, $P < 0.001$, different from RCS.

in females, where, however, very low expression levels of *Ar* occurred. Roughly the same pattern of *Ar* and *Er-α* expression occurred in preneoplastic liver of all strains. Both receptors were expressed at a lower level than in control liver, excepting *Ar* in females, which was expressed at very low levels, slightly lower than or no different from control values. *Er-β* gene expression was not detectable in all tissues tested.

The effect of feminization of male rats and masculinization of females on the development of preneoplastic liver was evaluated in RCS (Fig. 3, *right*). Normal and preneoplastic liver of male congenics exhibited a decrease in *Ar* and an increase in *Er-α* mRNA after orchiectomy plus β-estradiol and orchiectomy ± β-estradiol, respectively. In females, a relatively low increase in *Ar* and a sharp decrease in *Er-α* expression occurred in normal and preneoplastic liver after ovariectomy plus testosterone and ovariectomy ± testosterone, respectively. Concomitantly, gonadectomy resulted in a 1.5- to 2-fold decrease in serum testosterone and β-estradiol levels in congenic male rats 8 weeks after initiation (Table 1). Treatment with β-estradiol did not significantly affect testosterone but caused an ~3-fold increase in β-estradiol level. In female RCS, gonadectomy caused an ~2-fold decrease in β-estradiol and no changes in testosterone levels. Treatment with testosterone further decreased β-estradiol without influencing testosterone content. The alteration of hormone levels and receptor expression was associated with a decrease in PCNA index, intensified by β-estradiol administration, in liver lesions of gonadectomized males, and an increase in PCNA index, enhanced by testosterone, in gonadectomized females (Table 1). In addition, gonadectomy ± antagonistic hormone induced an increase in number and a decrease in volume and volume fraction of liver lesions in males, and an increase in volume and volume fraction without a change or decrease in number in females (Table 1; Supplementary Fig. S1).

Expression of candidate genes. We comparatively evaluated *Sc4mol, Cpe*, and *Klhl2* gene expression in normal and preneoplastic liver of RCS and parental strains, as well as the effect of sex

Table 1. Effect of gonadectomy and treatment with β-estradiol or testosterone on the serum levels of sex hormones and development of foci of altered hepatocytes in RCS

	Testosterone (ng/mL)	β-Estradiol (pg/mL)	PCNA index	Number/mm^3	Volume (cm^3 × 10^4)	Volume fraction
Male						
RCS	2.0 ± 0.50	12.4 ± 2.51	12.14 ± 1.45	3.84 ± 1.45	4.76 ± 0.40	59 ± 9.0
Orchiectomy	0.95 ± 0.21*	8.4 ± 2.01*	6.42 ± 0.99*	9.42 ± 0.99*	0.47 ± 0.13*	33 ± 8.0*
Orchiectomy + β-estradiol	0.85 ± 0.07*	25.7 ± 5.20*	3.55 ± 0.98*	16.82 ± 5,10*	0.18 ± 0.11*	19 ± 5.4*
Female						
RCS	0.44 ± 0.07 †	25.2 ± 4.21 †	2.76 ± 1.10 †	11.63 ± 0.89 †	0.16 ± 0.09 †	10 ± 4.1 †
Ovariectomy	0.40 ± 0.02	13.5 ± 3.70*	10.67 ± 1.40*	12.82 ± 2.80	0.39 ± 0.15*	19 ± 4.2
Ovariectomy + androgen	0.42 ± 0.02	5.0 ± 0.81*	15.33 ± 0.92*	7.38 ± 0.92*	3.90 ± 0.74*	69 ± 9.8*

NOTE: Liver lesions were induced by the "resistant hepatocyte" model in rats initiated by diethylnitrosamine. Two thousand to 4,000 GST 7-7(+) hepatocytes per liver were counted to determine PCNA index. PCNA index is expressed as the percentage of positive nuclei in liver lesions, and γ-glutamyl transpeptidase as enzymatic activity per gram of liver. Number, volume, and volume fraction of lesions were calculated by computer assisted analysis of GST 7-7(+) lesions. The results relative to RCS were taken from Fig. 2. Data are mean ± SD of five to nine rats.

*$P < 0.05$, different from RCS.

† $P < 0.01$, different from male RCS.

hormones in the congenic strain. As shown in Fig. 4 (*left*), *Sc4mol* expression was higher in normal liver of F334 than BN and congenic rats of both sexes. It sharply decreased in preneoplastic liver of F344 and BN rats, with low changes in congenic rats of both sexes. *Cpe* expression was higher in normal liver of male F344 than BN and congenic rats, and in liver of female F344 and BN rats compared with congenic rats. In preneoplastic liver, *Cpe* expression increased or did not change in male rats with respect to controls, whereas it decreased in F344 and BN female rats and slightly increased in congenic rats. The expression levels of *Klhl2* did not show any significant interstrain difference in normal and preneoplastic liver of male rats, whereas a slight decrease occurred in female congenic rats with respect to F344 and BN rats. *Klhl2* expression was significantly higher in preneoplastic than in normal liver of both male and female rats, with the highest values in females.

Feminization of male congenics by gonadectomy plus β-estradiol, in controls, and gonadectomy ± β-estradiol, in carcinogen-treated rats, resulted in *Sc4mol* and *Cpe* decreased expression (Fig. 4, *right*). Masculinization by gonadectomy plus testosterone or gonadectomy ± testosterone caused a sharp increase in *Cpe* and very small changes in *Sc4mol* expression in control and carcinogen-treated rats. No changes in *Klhl2* expression pattern were induced by hormonal manipulation in males, whereas gonadectomy ± testosterone induced a decrease in the expression levels in females.

Discussion

Genetic susceptibility to hepatocarcinogenesis of male F344, BN, and (BN × F344)F1 rats is under control of 7 *Hcs*, 9 *H*epato-*c*arcinogenesis *r*esistance (*Hcr*), and 2 *L*iver *n*eoplastic *n*odules *r*emodeling (*Lnnr*) loci, influencing the number, volume, and redifferentiation of neoplastic liver nodules, respectively (1). The presence of a *B* allele at *Hcs4* locus influences positively lesion volume (15). Our results reveal a sex dimorphism in HCC development in the resistant BN rats, analogous to that previously described in susceptible rat strains (5, 6). These findings also show that the presence of homozygous *B* alleles, between D16Mit5 and D16Rat75 at RNO16 in an isogenic *FF* background, is associated, in male congenic F344.BN-*Hcs4* rats, with a susceptibility to hepatocarcinogenesis comparable to that of male F344 rats and, in female congenics, with a resistance comparable to that of female BN rats. The absence of major interstrain differences in the basal plasma levels of sex steroids seems to exclude their role in the different progression patterns of preneoplastic lesions in the rat strains examined. Interstrain comparison of the relative basal expression of sex hormone receptors did not reveal any clear relationship with interstrain differences in the genetic susceptibility to liver cancer. *Ar* and *Er-α* were expressed at roughly the same level in F344 and congenic rats of both sexes, suggesting a control of *Ar* and *Er-α* expression by regulatory mechanisms outside the *Hcs4* locus. Male and female BN rats exhibited low expression of both *Ar* and *Er-α*, which could implicate a reduction in protective

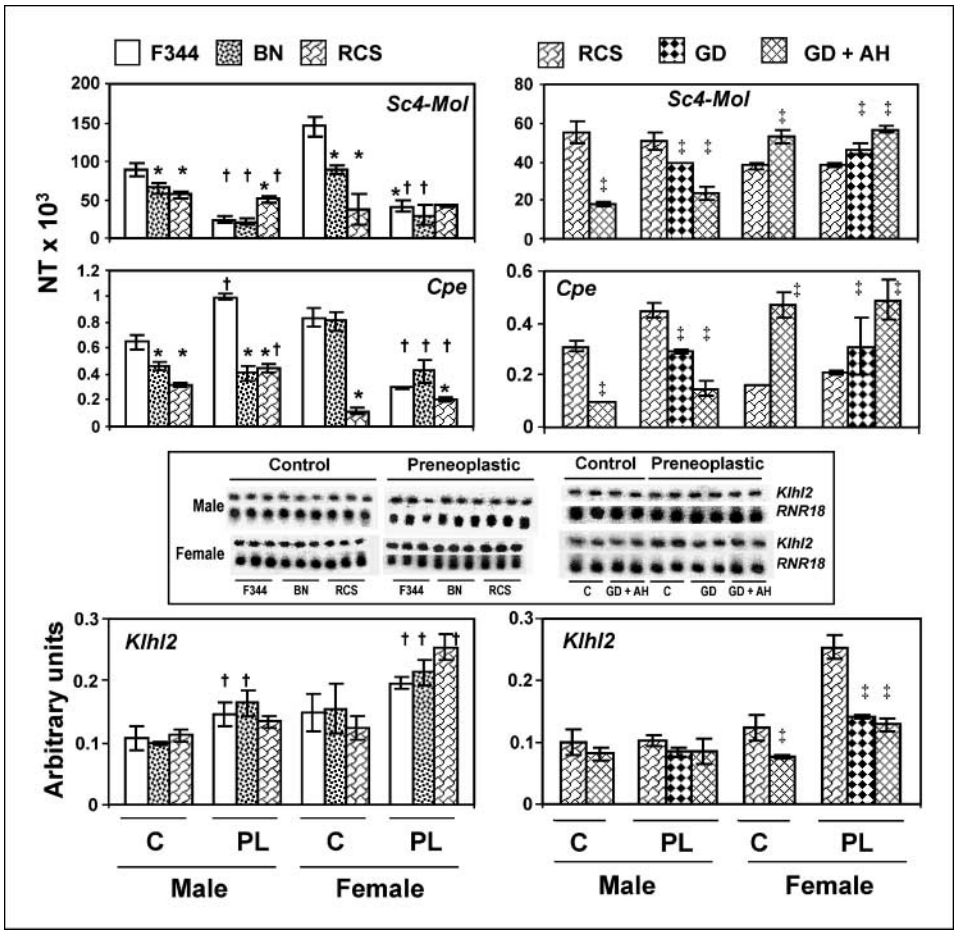

Figure 4. Expression of *Sc4mol, Cpe*, and *Klhl2* genes in normal and preneoplastic liver of F344, BN, and congenic (RCS) rats. When indicated, congenic rats were gonadectomized and treated with antagonistic hormones as described in Materials and Methods. *Columns,* mean of *N* target (NT) of three to seven rats (for quantitative RT-PCR), or relative amounts of four to six rats, with representative reproduction of RT-PCR radiolabeled products [for comparative (semiquantitative) RT-PCR]; *bars,* SD. NT = 2−ΔCt, where ΔCt value of the sample was calculated by subtracting the average Ct value of the target gene from the average Ct value of the *RNR-18* gene. Relative amounts were radioactive counts normalized to *RNR-18* values. *, $P < 0.05$, different from F344. †, $P < 0.001$, different from normal liver. ‡, $P < 0.01$, different from RCS.

effects exerted by female sex hormones on rat liver carcinogenesis (4–6), compensated by the promoting effects of androgens (20–22). A role of sex hormones on rat liver carcinogenesis was evidenced, at least in congenic rats, in stress conditions represented by gonadectomy and treatment with antagonistic hormones. This resulted in the acquisition of a susceptible phenotype in females and a resistant phenotype in males, as shown by the close correspondence of morphometric variables and PCNA values of male and female gonadectomized rats plus antagonistic hormones to those of female BN and male F344 rats, respectively. In addition, *Ar* and *Er-α* expressions were directly and inversely correlated, respectively, with growth rate of preneoplastic liver. This behavior clearly indicates a role of homozygous *B* alleles at *Hcs4* in the determination of the phenotypic patterns of female congenics. The existence of great differences among rats in the distribution of relatively few *B* alleles outside this locus excludes their role in the RCS phenotype. Thus, a plausible explanation for the phenotypic pattern of male and female congenic rats toward hepatocarcinogenesis may be the presence at *Hcs4* locus of a high penetrance gene(s), activated by estrogens and not influenced or even inhibited by testosterone, conferring resistance to female rats, and of which the BN allelic variant provides higher resistance.

The mechanisms whereby sex steroid hormones control hepatocarcinogenesis are poorly understood. The gene(s) of BN-*Hcs4* locus, responsible for the sex differentiation of susceptibility to hepatocarcinogenesis, is functionally different from mouse loci *Hcf1* and *Hcf2*, which abrogate inhibition of murine hepatocarcinogenesis by estrogens (14). *Hcf2* locus was recently shown to correspond to a potent modifier, *Hcs7*, located on distal mouse chromosome 1, which confers increased tumor multiplicity to a similar degree in male and female mice (23). Indeed, the strong resistance to hepatocarcinogenesis of BN and F344.BN-*Hcs4* females excludes the presence at *Hcs4* of a gene contrasting the inhibitory effect of estrogens on liver cancer development in female rats.

A number of genes, including *cyclin D1* and *c-myc*, are transactivated by estrogen-activated receptors (24), whereas proapoptotic genes of the Bcl family are down-regulated (25). Previous work has shown up-regulation of c-*myc* and *cyclin D1* genes in preneoplastic and neoplastic lesions induced in susceptible rat strains but not in resistant strains (16, 19, 26). Apoptosis is involved at a lower extent than redifferentiation of liver lesions in the acquisition of a resistant phenotype (15). Low growth rate, without increase in remodeling, characterizes the resistance of female and feminized males of RCS, which excludes an interference of this locus with the genes regulating the redifferentiation of liver lesions (1). It should be noted that cell cycle activity in neoplastic liver lesions of rat strains differently susceptible to HCC may be largely affected by differences in relative activities of stimulatory and inhibitory molecules involved in cell cycle control (16). Although our results suggest a possible interference of *Hcs4* locus with cell cycle overactivity in preneoplastic liver (16), further work is required to identify the molecular mechanisms determining the low growth capacity of preneoplastic liver in congenic rats.

Modifier genes responsible for the phenotypic effects of BN-*Hcs4* locus on growth and progression of preneoplastic liver are unknown. Numerous genes (potentially) related to hepatocarcinogenesis are located at this locus (Supplementary Table S2). However, there is no evidence for most of them of regulation by sex hormones. Indirect evidence of sex-linked regulation has been reported for *Sc4mol* and *Cpe* (27, 28). These genes and *Klhl2* are located at the telomeric portion of BN-*Hcs4*, syntenic to a portion of human chromosome 4, where allelic imbalance frequently occurs, envisaging the presence of a suppressor region (29, 30). *Sc4mol* encodes a sterol-4α-methyl oxidase that removes methyl groups at C4 of sterol molecules, allowing them to be further metabolized by decarboxylation followed by reduction (31). Thus, down-regulation of *Sc4mol* by estrogens could affect cholesterol precursors. Indeed, estrogens seem to inhibit cholesterol synthesis (27). The relationships between *Sc4mol* expression and tumorigenesis are unknown. However, the relatively low response of *Sc4mol* to β-estradiol seems to exclude its candidacy as a modifier gene responsible for inhibition of hepatocarcinogenesis in females. The data indicating regulation of *Cpe* expression by sex hormones are in agreement with the previous observation that Cpe protein production by the hypophysis of F344 rats is inhibited following diethylstilbestrol treatment (28). Cpe is involved in posttranscriptional maturation of insulin and enkephalin from their precursor polypeptides (32) and its role in the activation of growth regulatory factor may be hypothesized, but its involvement in liver tumorigenesis is unproved. *Klhl2* encodes a member of the family of actin binding proteins involved in the maintenance of cell polarity and mobility, regulation of cellular distribution of organelles and proteins, and mRNA transport from nucleus to cytoplasm (33, 34). Furthermore, Klhl2 induces c-Jun and cyclin D1 in breast cancer (35). Estrogens do not seem to affect the activity of *Klhl2*, which instead is presumably inhibited by testosterone. Overall, these results, together with the relatively low interstrain differences in the expression of *Sc4mol, Cpe*, and *Klhl2* and their deregulation in preneoplastic liver independent of liver volume occupied by preneoplastic lesions, speak against a role of these genes as cancer modifiers. However, this does not exclude that their activity is influenced by cancer modifier gene(s) at *Hcs4* locus involved in sex-differentiated hepatocarcinogenesis. A further restriction of *Hcs4 l*ocus in RCS is under way in an attempt to better characterize and clone the modifier gene responsible for sex dimorphism of hepatocarcinogenesis in the rat.

It is unknown if analogous genetic mechanisms determine sex dimorphism toward hepatocarcinogenesis in rats and humans. Although the possibility that estrogens enhance human hepatocarcinogenesis has been postulated (36), recent results failed to show a clear association between human HCC risk and plasma estradiol level and estradiol/testosterone ratio, whereas the risk of HCC was found to increase with plasma testosterone level (20). Variant forms of *Er* mRNA, lacking exon 5, probably associated with the loss of estrogen receptor responsiveness to estrogens, are expressed in human preneoplastic and neoplastic liver (37, 38), and the relative expression of these forms seems to be associated with ominous prognosis (39). Polymorphic human *ESR1* T29/T has recently been associated with persistent hepatitis B virus infection in a relatively small Chinese population (40). However, the functional status of the polymorphic receptor and its functional molecular mechanisms have not been investigated. Overall, different observations seem to indicate that estrogens inhibit hepatocarcinogenesis through the actions of functional receptors. Our results first identify a control of the sex-differentiated susceptibility of rats to HCC by a potent putative modifier gene(s) mapping to RNO16. This gene has not yet been

identified, and it is not clear if it is a suppressor gene directly influenced by sex hormones or acting cooperatively with sex hormone–sensitive genes. If a hormone-sensitive modifier gene, homologous to that of rats, is present in human genome, it could be envisaged the possibility that mutations abolishing inhibition by β-estradiol of this gene contribute to the development of liver cancer in women.

Acknowledgments

Received 8/3/2006; revised 9/6/2006; accepted 9/7/2006.

Grant support: Associazione Italiana Ricerche sul Cancro, Fondazione Italiana per la Ricerca sul Cancro, Fondazione Compagnia di San Paolo, Ministero dell'Istruzione, Università e Ricerca, and Assessorato Igiene e Sanità RAS.

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Mapping a Sex Hormone–Sensitive Gene Determining Female Resistance to Liver Carcinogenesis in a Congenic F344.BN-*Hcs4* Rat

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Cancer Res 2006;66:10384-10390.