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The *Hcs7* Mouse Liver Cancer Modifier Maps to a 3.3 Mb Region Carrying the Strong Candidate *Ifi202b*

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1. Introduction

Genes that affect a person's chance of developing hepatocellular carcinoma (HCC), as *BRCA1* and *BRCA2* affect a person's chance of developing breast or ovarian cancer, have been difficult to detect. The vast majority of liver cancers can be attributed to Hepatitis B or C virus infection, aflatoxin exposure, or alcoholic cirrhosis, alone or in combination (Montalto et al., 2002). This high background of predisposing environmental factors makes identifying less penetrant genetic contributors more difficult. Familial patterns of susceptibility to liver cancer independent of environmental factors have helped identify a few monogenic metabolic syndromes (*e.g.*, hemochromatosis; Dragani, 2010). However, the analysis of liver tumors points to a variety of other genes that affect liver tumor development. The patterns of chromosome gain and loss in liver cancers worldwide reveal several regions that are gained or lost in up to 86% of tumors, including gains of 1q, 6p, 8q, and 20q, and losses of 1p, 4q, 6q, 8p, 13q, 16q, and 17p (Lau and Guan, 2005; Chochi et al., 2009; Zhang et al., 2010). Chromosome analyses combined with genome-wide association studies have revealed candidate HCC modifier genes for some of these regions, such as *PAPSS1* on chromosome 4q and *HCAP1* on chromosome 17p (Wan et al., 2004; Shih et al., 2009).

Mice present an independent model of liver carcinogenesis for which external variables such as chemical exposure can be controlled and the genetics is manipulable. Different inbred strains of mice develop cancers at different frequencies. Because these differences are genetic, and the mice are homozygous due to inbreeding, causative genes can be identified through positional cloning.

Inbred mouse strains differ dramatically in their susceptibility to both spontaneous and carcinogen-induced HCC. Females of the C57BR/cdJ strain, for example, develop up to 50-fold more tumors after a single injection of N,N-diethylnitrosamine (DEN) than females of the related, relatively resistant C57BL/6J (B6) strain. We have recently mapped the

predominant locus responsible for this difference to a 6 Mb region on Chromosome 17 (Peychal et al., 2009). This region corresponds to part of the chromosome 6p region amplified in the majority of late-stage HCC (Santos et al., 2007; Chochi et al., 2009).

The C3H/HeJ strain (C3H), highly susceptible to both spontaneous and carcinogen-induced HCC, develops up to 50-fold more liver tumors than the B6 strain after a single carcinogen treatment (Drinkwater and Ginsler, 1986). We previously reported mapping the predominant locus responsible for this susceptibility, *Hcs7*, to distal Chromosome 1 (Bilger et al., 2004). This chromosomal region corresponds in part to the 1q21-24 chromosomal region amplified in up to 86% of human liver cancers (Lau and Guan, 2005; Chochi et al., 2009; Zhang et al., 2010). C3H alleles on mouse Chromosome 1 confer a dominant 15-fold increased susceptibility to male mice and a semi-dominant 5-fold increased susceptibility to female mice carrying C3H alleles (Bilger et al., 2004).

Here we analyze the effect of C3H Chromosome 1 alleles on spontaneous hepato-carcinogenesis, on apoptosis and mitosis after DEN treatment, and on preneoplastic lesion growth. We have mapped the *Hcs7* modifier to a 3.3 Mb region and used expression and CGH arrays to identify the *Ifi202b* gene as a strong candidate for this locus.

2. Materials and methods

2.1 Mice

B6 and C3H mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and bred in our facilities. All mice were housed in plastic cages on corncob bedding (Bed O'Cobs, Anderson Cob Division, Maumee, OH), fed Purina 5020 diet (9% fat; St. Louis, MO), and given acidified tap water *ad libitum*. Mice were inspected daily and weighed monthly.

Lines derived from the B6.C3H-Ch1 recombinant strain (Bilger et al., 2004) were generated as follows. B6 females were mated with B6.C3H-Ch1 males heterozygous for the congenic region. Markers between *D1Mit285* and *D1MIT17* were used to identify recombinants, and breakpoints were mapped more finely with additional markers. Males and females from a given recombinant line were then intercrossed to generate homozygotes. Heterozygous or homozygous mice were mated with B6 to generate heterozygous experimental progeny (Figures 1 and 3, and Table 3) or homozygotes were intercrossed to generate homozygous experimental progeny (Tables 1 and 2). Tested parental B6.C3H-Ch1 were homozygous.

Lines derived from B6.BR-Ch1 were generated by mating B6 females with B6.BR-Ch1 males heterozygous for the congenic region. The markers *D1Mit143*, *D1Mit17*, and two additional microsatellite markers at 172.9 and 176.9 Mb were used to identify recombinants. (The lack of SNPs or SSLPs between B6 and BR in this region prevented refinement of these breakpoints.) Heterozygotes were intercrossed to generate homozygous lines. B6 females were then crossed with homozygous congenic males to generate experimental progeny.

The C3H.B6-Ch1 line carrying B6 alleles for distal Chromosome 1 on a C3H genetic background was generated by crossing C3B6F1 (N1) mice with C3H mice and selecting C3H.B6 N2 mice carrying B6 alleles at *D1Mit143*, *D1Mit15*, *D1Mit166*, and *D1Mit461*. Backcrossing to C3H and selection of these B6 alleles was continued for eight additional generations to generate C3.B6(*D1Mit143-D1Mit461*)N10, or "C3H.B6-Ch1" (Figure 3) mice.

2.2 Genotyping

DNA was prepared from spleen tissue by Proteinase K treatment/ammonium acetate/isopropyl alcohol precipitation as described (Bilger et al., 2004) or from spleen, tail, or toe tissue by alkaline lysis (modified from Truett et al., 2000). Approximately 2 mm^3 of spleen or 10 mm^3 of tail or toe tissue was incubated in 200 µl lysis solution (25 mM NaOH, 0.2 mM EDTA) at 95°C for 20-60 minutes and then neutralized by the addition of 200 µl 40 mM Tris (pH 8.0). After agitation for approximately 30 seconds and centrifugation for five minutes at 13.5 krpm, 0.25 to 2 µl of the supernatant was used directly for genotyping as described (Bilger et al., 2004).

2.3 Tumor and preneoplastic lesion induction and assessment

Tumors were induced by a single intraperitoneal injection of DEN (Eastman Kodak Co., Rochester NY; 0.1 µmol/g body weight) dissolved in tricaprylin (a.k.a. trioctanoin; Sigma, St. Louis, MO) 12 ± 1 days after birth. Mice, all male, were sacrificed by CO_2 asphyxiation. For prenoplastic lesion volume analysis, mice were sacrificed at 16 or 24 weeks of age (Table 2) and representative sections of the liver were frozen immediately on dry ice and stained for glucose-6-phosphatase activity as described (Bugni et al., 2001). Glucose-6-phosphatase deficiency is a hallmark of the majority of preneoplastic lesions in DEN-treated mice (Bugni et al., 2001). For tumor analysis, mice were sacrificed at 32 weeks of age (Figure 1, Figure 3). Livers were removed and weighed, and all tumors larger than 1 mm in diameter were counted. Liver tumors were sampled at random, fixed in RNAlater (Qiagen, Valencia, CA) at 4°C, then drained of RNAlater and transferred to -80°C. Spleens were collected as a source of DNA and frozen on dry ice. All tumors were scored by a single observer blind to genotype. To assess spontaneous tumors (Table 1), mice were allowed to age to 15 months without undergoing any procedures. Tumors larger than 1 mm in diameter were counted.

To assess apoptosis, mitosis, and RNA expression immediately following DEN injection, male mice were either left untreated or injected with DEN (0.1 µmol/g body weight), at 12 days of age between 11 a.m. and 3 p.m.. Mice were sacrificed one, two, or three days later between 3 and 6 p.m.. One half of each left lobe and half of each half of the medial lobe were placed in formalin at 4°C. Half of the remaining parts of the left and medial lobes were placed in RNAlater at 4°C. The remaining portions of the left and medial lobes were frozen in pre-chilled tubes on dry ice and transferred to -80°C.

Formalin-fixed tissue was sectioned and stained with hematoxylin and eosin (H&E). Hepatocytes, apoptoses, and mitoses were counted under 600× magnification.

2.4 Microarray analysis of gene expression

RNA was purified from 20-70 mg liver or liver tumor tissue fixed in RNAlater using an RNeasy MIDI kit (Qiagen, Valencia, CA) as directed, except 50% ethanol, rather than 70% ethanol, was added to the homogenized lysate. Fluorescently labeled cDNAs were generated using Agilent Quick-Amp Labeling kits and hybridized in competition with a mixed-sex, whole-tissue liver cDNA control to Agilent Whole Mouse Genome arrays (Item #G4122F, Agilent, Santa Clara, CA). Arrays were scanned using an Agilent DNA Microarray Scanner G2505C.

2.5 Comparative genomic hybridization

Genomic DNA from male spleens was prepared using a standard Proteinase K/ammonium acetate/isopropyl alcohol precipitation protocol as described (Bilger et al., 2004), except DNA was treated with RNase A, which was then precipitated with ammonium acetate before the final DNA precipitation. Genomic DNA of either line 1R5 or inbred C3H mice was then labeled and mixed with control genomic B6 DNA, for dual-color CGH, and hybridized to the MM 8_WG_CGH_1of8 chip. This array has 50-75 nucleotide probes spaced at an average of 650 bp, covering all of Chr 1 and Chr 2 through position 123959568. Results were analyzed by Nimblegen CGH Services, using the CGH-segMNT algorithm (Roche Nimblegen, Madison, WI).

2.6 Statistical analysis

The significance of differences between tumor multiplicity data sets was determined by the Wilcoxon rank-sum test, using Mstat software (version 5.4, McArdle Laboratory for Cancer Research; URL, http://www.mcardle.wisc.edu/mstat). All *P*-values were calculated on the basis of a two-sided test, except in the case of spontaneous tumorigenesis, for which we were testing the one-sided hypothesis that C3H alleles that increase DEN-induced tumor multiplicity also increase spontaneous tumor multiplicity. Differences between RNA expression data sets were evaluated with the limma software package (Smyth, 2004), using a moderated t-statistic followed by FDR correction.

3. Results

3.1 A dominant modifier on Chr 1, *Hcs7*, maps to a 3.3 Mb region between 175.35 and 178.64 Mb

To map the Chromosome 1 modifier to a smaller region, we crossed B6 mice with B6.C3H-Ch1 congenic mice carrying *Hcs7* susceptibility alleles on Chromosome 1. Mice that had undergone recombination in the susceptibility region were selected and then bred again with B6 mice to generate heterozygous progeny cohorts for phenotyping. Male progeny were injected with DEN at 12 days and sacrificed at 32 weeks of age. The median number of tumors developed by males of each recombinant line is shown in Figure 1, below.

Most of the susceptibility conferred by *Hcs7* can be mapped to the distal end of the chromosome. Lines carrying C3H alleles only distal of *D1Mit143* at 165 Mb developed 5- to 12-fold more tumors than B6 (line 1R28: $P < 10^{-7}$; line 1R33: $P < 10^{-6}$; line 1R42: $P < 10^{-5}$). For lines 1R28 and 1R33, the number of tumors developed was not significantly different from the number developed by the homozygous B6.C3H-Ch1 parental congenic line (1R28: $P > 0.30$; line 1R33: $P > 0.11$).

Six of the seven recombinant lines that carry C3H from *D1Mit15* at 170 Mb to *D1Mit17* at 191 Mb near the telomere were highly susceptible. These six lines developed an average of eight times as many tumors as B6. The exception, line 1R5, carries this susceptibility region but was resistant (1R5 vs B6: $P > 0.35$). Comparative genomic hybridization revealed a potential explanation. In the distal susceptibility region, line 1R5 differs from C3H at the 5' end of the *Ifi202b* gene (Figure 2). While line 1R5 and C3H both carry polymorphisms that suggest duplication of part of this region relative to B6, line 1R5 also carries polymorphisms that reduce hybridization to the CGH probes relative to B6 and C3H. This effect could be due to deletion, rearrangement, or novel sequences.

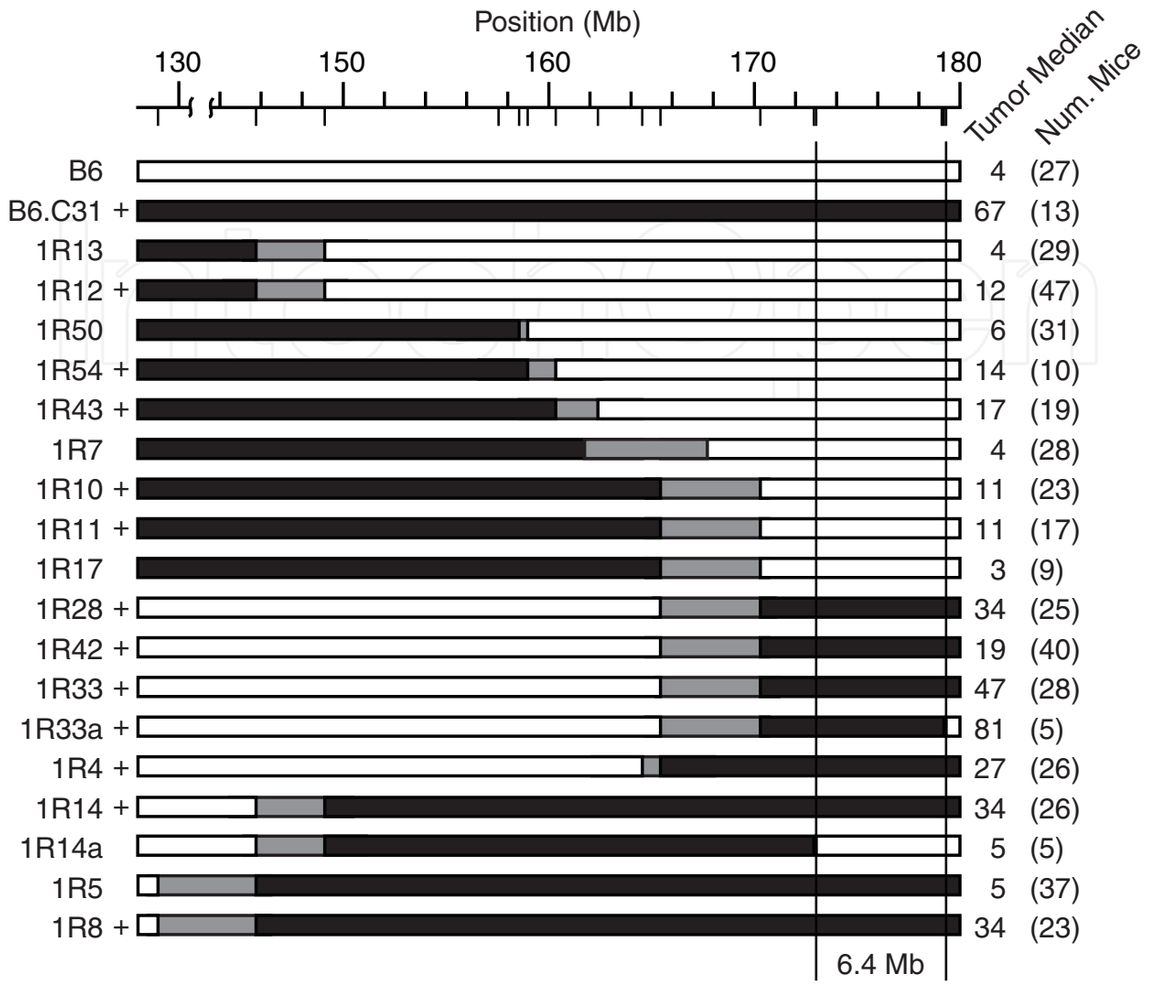

Fig. 1. C3H liver cancer susceptibility alleles map to distal Chromosome 1.

Male mice carrying C3H alleles on Chromosome 1 on a B6 genetic background were injected with DEN and liver tumors were counted at 32 weeks. The line designated B6.C31 refers to B6.C3H-Ch1. Regions inherited from B6 are shown in white; C3H regions are shown in black. Regions carrying a breakpoint between B6 and C3H alleles are shown in grey. The median tumor multiplicity and number of mice tested are shown to the right of each line. Lines that were significantly more susceptible than B6 are marked with a "+." The positions of markers along Chromosome 1 are shown as tics below the position axis at the top of the figure.

Two additional lines, derived from the susceptible 1R14 and 1R33 lines during the breeding of experimental progeny, suggested that *Hcs7* lies in a 6.4 Mb region carrying *Ifi202b*. Although few mice were phenotyped (five per line), the results for each line were significant. Line 33a, like its parent, was significantly more susceptible than B6 ($P < 10^{-3}$) and not significantly different from B6.C3H-Ch1 ($P > 0.55$). The distal breakpoint for line 1R33a is proximal of 179.3 Mb, suggesting that the minimal susceptibility region lies proximal of 179.3 Mb. Line 1R14a, unlike its parental line 1R14, was resistant to hepatocarcinogenesis. The difference between the lines is the distal breakpoint between 172.9 and 173.0 Mb in line

1R14a, suggesting that the minimal susceptibility region is distal of 172.9 Mb. Together, the two sublines suggest that *Hcs7* lies between 172.9 and 179.3 Mb.

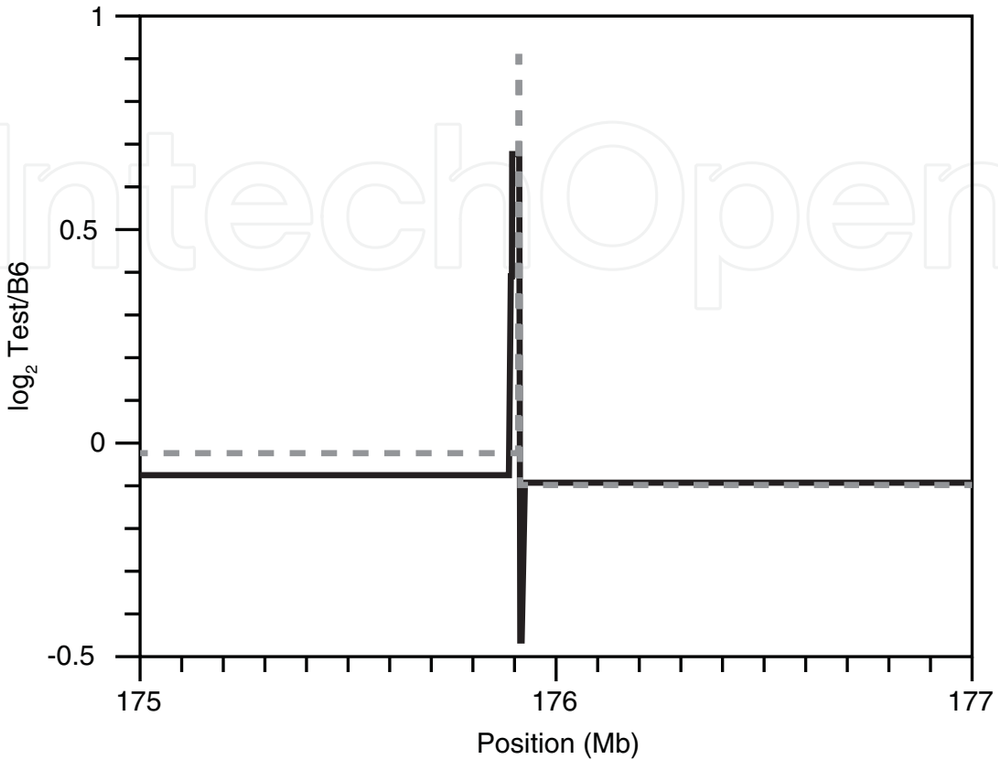

Fig. 2. The homozygous line 1R5 genome differs from C3H at *Ifi202b*.

Genomic DNA of either line 1R5 or inbred C3H was labeled and mixed with control genomic B6 DNA before hybridization to a chip carrying Chromosome 1 probes spaced 650 bp apart. The $\log_2$ ratio of the mean of the intensity of the 1R5 or C3H signal (Test) relative to B6 is plotted. C3H is shown as a dashed grey line; the 1R5 recombinant is shown as a solid black line.

Several other congenic lines that lacked the distal susceptibility region were significantly more susceptible than B6, developing three- to four-fold more tumors by 32 weeks (lines marked with plus signs, Figure 1; $P < 10^{-3}$ for all). These lines and the closely related congenic lines that are as resistant as B6 reveal a complex pattern of possibly interacting modifiers along proximal Chromosome 1. Resistant line 1R50, for example, carries more C3H than sensitive line 1R12, but it carries less C3H than sensitive line 1R54 (1R50 vs B6: $P > 0.06$; vs R12: $p < 10^{-2}$; vs 1R54: $p < 0.02$). Similarly, the C3H region carried by resistant line 1R7 extends farther distally than the C3H region carried by sensitive line 1R43. However, the resistance of line 1R7 might be explained by a unique B6 genotype at the proximal end of Chromosome 1 (proximal of 74 Mb).

To localize the distal Chromosome 1 liver cancer modifier further, we bred line 1R33 with B6 and selected males with breakpoints in or near the region between 172.9 and 179.3 Mb (Figure 3). These mice were bred with B6 females to generate experimental progeny that were injected with DEN at 12 days; tumors were counted at 32 weeks.

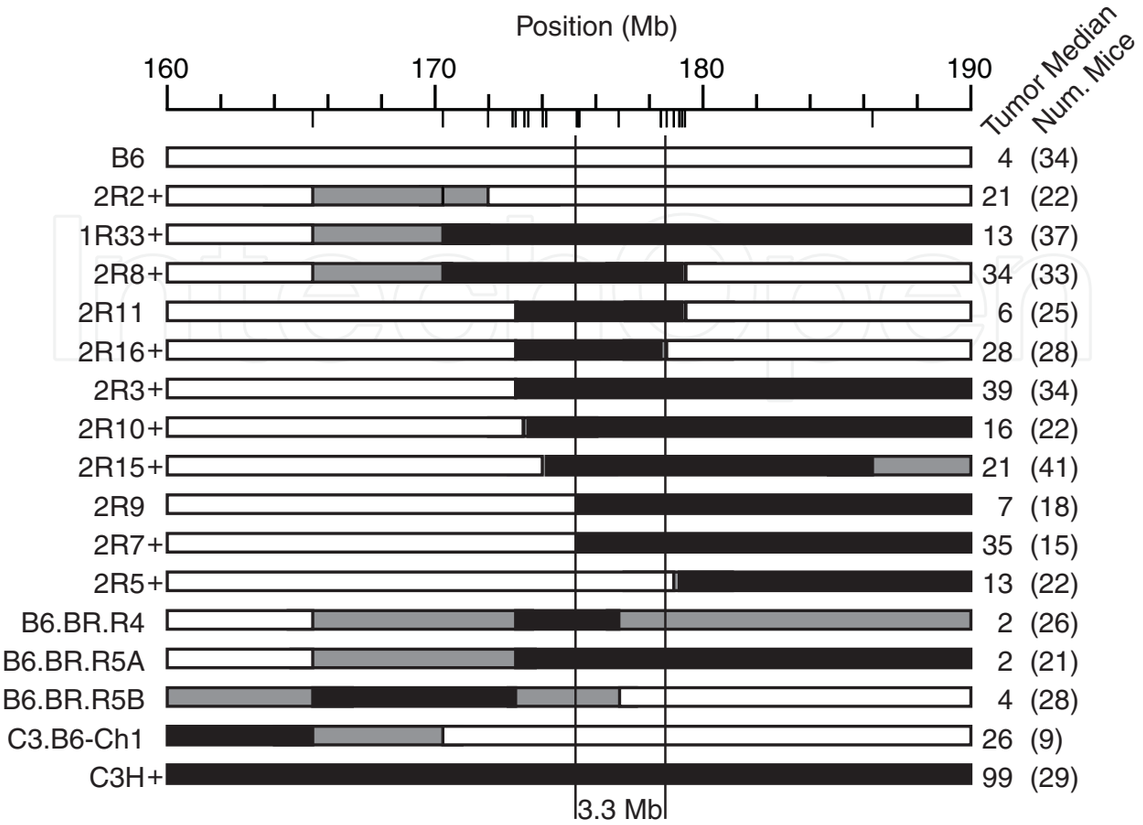

Fig. 3. C3H liver cancer susceptibility alleles map to a 3.3 Mb region between 175.35 and 178.64 Mb.

Male mice were injected with DEN and liver tumors were counted at 32 weeks. "B6" and "C3" mice were inbred; lines beginning with a "1" or "2" were B6.C3 congenic mice carrying C3H Chromosome 1 alleles on a B6 genetic background; "B6.BR." lines carry BR Chromosome 1 alleles on a B6 genetic background; and the C3H.B6-Ch1 (C3.B6-Ch1) line carries B6 Chromosome 1 alleles on a C3H background. Regions inherited from B6 are shown in white; C3H and BR regions are shown in black. Regions carrying a breakpoint between B6 and C3H alleles, or between B6 and BR alleles, are shown in grey. The median tumor multiplicity and number of mice tested are shown to the right of each line. Lines that were significantly more susceptible than B6 are marked with a "+." The positions of markers along Chromosome 1 are shown as tics below the position axis at the top of the figure.

Mice from line 2R16, which carries the smallest susceptibility region, developed 7-fold more tumors than B6 ($P < 10^{-5}$; Figure 3). This line carries C3H from 172.9 to 178.64 Mb, confirming and narrowing the location of the distal modifier. Line 2R16 was derived from line 2R3, which carries an additional ~14 Mb of C3H alleles distal of 178.64 Mb. Like 2R16, 2R3 was highly susceptible, developing 10-fold more tumors than B6 (line 2R3 vs. 2R16: $P > 0.12$; vs. B6: $P < 10^{-8}$). Line 2R8 has a larger congenic region than 2R16, extending up to 7.5 Mb farther proximally and ~ 700kb farther distally. This line was also highly susceptible, developing more than 8-fold more tumors than B6 ($P < 10^{-5}$). Together with the parental line 1R33 (again highly significantly different from B6), these lines establish that the *Hcs7* locus

confers a 7- to 10-fold increase in tumor multiplicity and lies between 172.9 and 178.64 Mb. This region carries 132 genes (www.ensembl.org; 8/2011). Line 2R7, which extends distally from 175.35 Mb, was also highly susceptible relative to B6, developing almost 9-fold more tumors ($P < 10^{-5}$). In addition, line 2R7 is approximately 3-fold more susceptible than line 2R5, which carries C3H alleles distal of 178.9 Mb ($P < 0.02$). Together with the 2R16 results that place *Hcs7* between 172.9 and 178.64 Mb, these data suggest that *Hcs7* lies in the 3.3 Mb between 175.35 and 178.64 Mb. This region carries 44 genes (www.ensembl.org; 8/2011).

Again, the remaining lines suggest that the pattern of modifiers along the chromosome is complex. Lines 2R9 and 2R7 both carry C3H alleles to near the telomere and line 2R9 carries more C3H alleles than 2R7 proximally (their breakpoints differ by about 100 Kb), but line 2R9 is resistant (2R9 vs B6: $P > 0.24$). Similarly, the resistance of line 2R11 (2R11 vs. B6: $P > 0.09$), which, like line 2R9, was derived from sensitive line 2R3, together with the susceptibility of lines 2R2, 2R10, 2R15, 2R7, and 2R5, suggests there are additional modifiers both proximal to and distal of the 175.4 to 178.64 Mb minimal region. Alternatively, some lines may have undergone rearrangement in the susceptibility region.

Recombination between B6 and C3H was suppressed in the minimal susceptibility region, between 175.4 and 178.4 Mb. No recombinants were observed among approximately 1350 segregating progeny, although 24 would be expected. This difference is highly significant ($P < 10^{-6}$). Recombination is frequently suppressed by chromosomal rearrangements such as inversions (Kirkpatrick, 2010).

3.2 B6 alleles on distal Chr 1 are sufficient to suppress hepatocarcinogenesis on a C3H background

To determine whether B6 Chromosome 1 alleles can confer resistance to a sensitive C3H background (as C3H alleles confer sensitivity to a resistant B6 background), we generated C3H.B6-Ch1 congenics using ten generations of backcrossing B6 to C3H, selecting B6 alleles on distal Chromosome 1 at each generation. Heterozygous and homozygous congenic males and control inbred C3H males were injected with DEN at 12 days of age and their tumors were counted at 32 weeks (Figure 3). While heterozygosity for B6 alleles had no effect on tumorigenesis ($P>0.84$), homozygosity caused a 3.8-fold reduction in tumor multiplicity ($P<10^{-3}$).

3.3 C57BR/cdJ alleles between 175.35 and 178.64 Mb do not confer susceptibility

We have shown previously that distal Chromosome 1 carries a modifier that causes 5- to 6-fold increased susceptibility in male C57BR/cdJ (BR) mice relative to B6 mice (Poole and Drinkwater, 1996; Bilger et al., 2004). To determine whether this *Hcif2* (formerly *Hcf2*) modifier might involve the same locus that confers susceptibility to C3H, we used B6.BR-Ch1 congenic mice to derive mice carrying smaller congenic regions and counted their tumors at 32 weeks (Figure 3). The line carrying the largest BR region, B6.BR.R4, developed fewer tumors than B6 (though not significantly; $P > 0.37$). Similarly, lines B6.BR.R5A and B6.BR.R5B, which carry part of the minimal region and extend distally beyond 191 Mb (R5A) or proximally beyond 165 Mb (R5B), also developed the same number of tumors as B6 or fewer ($P > 0.86$ and $P > 0.68$, respectively). All three recombinant lines developed significantly fewer tumors than the parental B6.BR-Ch1 line (not shown; $P < 0.03$ for all). These results indicate that *Hcif2* is unlikely to lie in the 175.35 to 178.64 *Hcs7* region.

3.4 Haplotype analysis of sensitive and resistant strains

The CBA/J strain is a cousin to C3H. Like its relative, CBA/J is highly susceptible to liver tumorigenesis, and the dominant modifier mapped in crosses between CBA/J and B6 maps to distal Chromosome 1 (Bilger et al., 2004). Because these strains are likely to share a susceptibility allele, we generated a haplotype map of the minimal *Hcs7* region in CBA using SNPs obtained through the Imputed SNP Database (Szatkiewicz et al., 2008). Only SNPs that could be determined with greater than 80% confidence were included (Figure 4). This map suggests that CBA is highly related to the C3H strain through most of the *Hcs7* region, confirming the likelihood that these two sensitive strains share one or more susceptibility alleles.

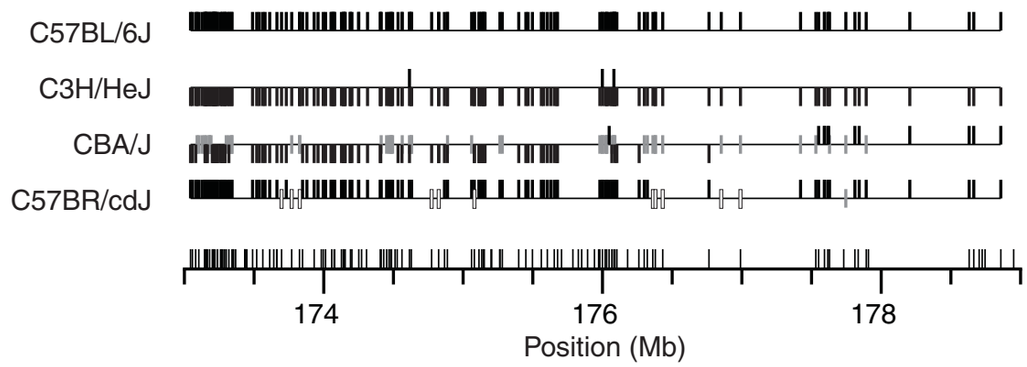

Fig. 4. Haplotype maps for the *Hcs7* interval.

Haplotypes were determined for each gene and intergenic region. B6 haplotypes are shown as tics above the bar. C3H haplotypes are shown as tics below the bar. Haplotypes unique to CBA are shown as grey tics centered on the bar, while haplotypes unique to BR are shown as outlined tics centered on the bar. The starting position of each gene is shown as a tic above the position axis.

3.5 C3H Chromosome 1 alleles increase spontaneous hepatocarcinogenesis

To determine whether *Hcs7* influences spontaneous as well as DEN-induced hepatocarcinogenesis, homozygous male mice were aged to 15 months and tumors were counted (Table 1). B6.C3H-Ch1 males developed significantly more liver tumors than B6 males (one-sided $P < 0.01$). The 4-fold higher multiplicity and the 2.5-fold higher incidence in this line closely resemble the higher multiplicity and incidence seen in inbred C3H males ($P > 0.85$). Similarly, line 1R33 males developed twice as many tumors and had 50% higher incidence than B6. The difference in tumor multiplicity was significant (one-sided $P < 0.04$). Males of the proximally congenic line 1R11, which developed approximately 3-fold more tumors than B6 when treated with DEN, had approximately 25% higher incidence and developed approximately 25% more spontaneous tumors than B6, but this difference was not significant (one-sided $P > 0.18$). This analysis of spontaneous tumor incidence indicates that the majority of the difference between C3H and B6 can be ascribed to alleles on Chromosome 1, including alleles in the *Hcs7* region.

Line	N	Incidence	Multiplicity
B6	46	0.21	0.28 ± 0.58
C3H	38	0.53	0.84 ± 1.10
B6.C3H-Ch1	17	0.47	1.35 ±2.21
1R33	31	0.35	0.65 ± 1.05
1R11	44	0.25	0.34 ± 0.71

Table 1. B6.C3H-Ch1 and 1R33 males are more susceptible to spontaneous liver tumors than B6.

Inbred and recombinant male mice were left untreated to 15 months of age and liver tumors were enumerated. N, the number of mice per group.

Many line 1R33 males developed severe skin disease. Twenty-eight males died or were euthanized prior to the 15-month time point (as compared to three B6, eight C3H, ten B6.C3H-Ch1, and eight line 1R11 males). These line 1R33 males developed skin problems with a frequency that differed from both B6 and the B6.C3H-Ch1 line, suggesting that multiple cooperating genes along Chromosome 1 must be derived from the same strain to prevent skin lesions. *Ifi202b* and neighboring genes have been implicated in autoimmune skin disease in distal Chromosome 1 congenic lines derived from B6 and the NZB (New Zealand Black) strain (Choubey et al., 2010; Panchanathan et al., 2011).

3.6 *Hcs7* affects early lesion growth

To determine when *Hcs7* influences tumorigenesis, we measured preneoplastic lesion size in B6, C3H, and congenic lines that had been treated with DEN at 12 days of age. Livers were collected at 16 and 24 weeks and frozen immediately on dry ice, followed by glucose-6-phosphatase staining to identify preneoplastic lesions. In the first experiment, lesions were measured in B6, line 1R11, and line 1R33 livers. By sixteen weeks, lesions in the moderately susceptible line 1R11 and the highly susceptible line 1R33 occupied 3.5 and 3.4 times, respectively, the volume occupied by B6 lesions ($P < 10^{-3}$ for both lines; Table 2). At 24 weeks, 1R11 lesions occupied 1.5-fold more volume than B6 lesions ($P > 0.15$), while 1R33 lesions occupied 2.0-fold more volume than B6 lesions ($P < 0.05$). This result indicates that the growth advantage of lesions in the congenic lines begins prior to 16 weeks.

A second experiment yielded results for line 2R8 that were very similar to those obtained for the larger 1R33 congenic. Line 2R8 lesions occupied 3.4-fold more volume than B6 lesions at 16 weeks; the ratio was 3.3-fold at 24 weeks. This result confirms that *Hcs7* modifies preneoplastic lesion growth. This second experiment included C3H males, which developed lesions that occupied significantly more volume than the congenic at both time points (16 wks: $P < 0.01$; 24 wks: $P < 10^{-4}$). C3H lesions occupied 17-fold more volume than B6 at 16 weeks, which increased to 33-fold more than B6 at 24 weeks. The magnitude of the growth effects in inbred C3H males suggests that *Hcs7* C3H alleles are not sufficient to recapitulate entirely the early lesion development seen in C3H inbred mice.

Experiment/age	N	Focus Volume Fraction (VF) ± SD	VF ratio
Experiment 1			
16 weeks			
B6	14	0.00098 ± 0.0011	-
1R33	19	0.0033 ± 0.0020	3.4
1R11	10	0.0034 ± 0.0022	3.5
24 weeks			
B6	18	0.022 ± 0.029	-
1R33	12	0.043 ± 0.040	2.0
1R11	9	0.033 ± 0.040	1.5
Experiment 2			
16 weeks			
B6	13	0.00071 ± 0.00062	-
2R8	12	0.0024 ± 0.0016	3.4
C3H	12	0.012 ± 0.011	17
24 weeks			
B6	12	0.0042 ± 0.0042	-
2R8	12	0.014 ± 0.012	3.3
C3H	11	0.14 ± 0.093	33

Table 2. Distal Chromosome 1 alleles influence net lesion growth before 16 weeks.

Male mice were treated at 12 days of age with DEN and sacrificed at 16 or 24 weeks of age. Frozen liver sections were evaluated for the presence and size distribution of glucose-6-phosphatase-deficient foci. N, number of animals per group; VF ratio, volume fraction ratio relative to B6.

3.7 *Hcs7* does not modify apoptosis or mitosis in the acute response to DEN injection

Other modifiers of DEN-induced hepatocarcinogenesis, such as IL-6 and IKKgamma, have been shown to affect apoptosis and/or mitosis within 72 hours of DEN administration (Maeda et al., 2005; Naugler et al., 2007). To determine whether the tumor susceptibility conferred by the *Hcs7* region affects mitosis or apoptosis rates during this acute response to DEN injection, we collected livers from line 2R8 males one, two, or three days after DEN injection at 12 days of age. Livers were fixed in formalin and stained with H&E. Mitoses and apoptoses were scored based on staining and morphology, under 600× magnification. Two microscopic fields centered on a portal vein and two fields centered on a central vein were scored. Hepatocytes were counted for 10-25 fields per strain and condition (*e.g.*, B6/DEN). Each field represented an equivalent number of hepatocytes per field (~330) in resistant and susceptible mice, independent of DEN treatment ($P > 0.07$ for all comparisons).

Rates of mitosis and apoptosis never differed significantly between B6 and line 2R8, whether or not mice were treated with DEN ($P > 0.10$ for all comparisons; Table 3). These results

indicate that the tumorigenic effect of *Hcs7* does not involve acute changes in the rates of apoptosis or mitosis in response to DEN injection.

		Num. mice		Mitoses / field		Apoptoses / field	
Age	**Treatment**	**B6**	**2R8**	**B6**	**2R8**	**B6**	**2R8**
13 days	No DEN	7	11	1.50	1.25	0.50	0.50
14 days	No DEN	9	9	2.00	1.5	0.25	0.50
15 days	No DEN	7	8	1.40	1.00	0.30	0.25
13 days	DEN	7	7	0.5	0.5	0.30	0.25
14 days	DEN	9	11	2.25	2.50	0.25	0.50
15 days	DEN	7	12	2.00	1.85	0.50	0.55

Table 3. Mitosis and apoptosis do not differ significantly between line 2R8 and B6 during tumor initiation.

3.8 The *Hcs7*-interval genes most differentially expressed between C3H and B6 are immune genes

To identify potential candidate genes and pathways regulated by the *Hcs7* region, we collected livers from DEN-treated B6 and 2R8 mice at 13 days, 28 hours after DEN injection. RNA from these livers was reverse-transcribed and hybridized to Agilent whole-genome arrays, in competition with a B6 mixed-sex control, to identify differentially expressed genes. These arrays carry 43,379 probes representing most genes in the genome. Of the 44 genes in the 175.35 to 178.64 Mb region, 35 are represented by probes. The remaining nine include genes for three spliceosomal RNAs, two olfactory receptors, one protein of unknown function, and three pyrin-domain-containing genes. Genome-wide, *Ifi202b* is the only significantly differentially expressed transcript based on FDR correction ($q < 10^{-2}$). The expression of *Ifi202b* is 57-fold higher in line 2R8 than in B6.

To identify potential candidates that may not be expressed at 13 days, RNA from three B6 and three 2R8 tumors was reverse transcribed and hybridized individually in competition with the mixed-sex control DNA. Genome-wide, *Ifi202b* was again the only significantly differentially expressed transcript, with levels 48-fold higher in 2R8 tumors than in B6 tumors (genome-wide FDR $q < 0.05$). When limiting analysis of the expression array data set to only the 35 candidate genes in the 175.35 to 178.64 Mb minimal region, both *Ifi202b* and the closely related *Aim2* (*Absent in melanoma 2*) were expressed at significantly different levels ($q < 10^{-4}$ and $q < 10^{-2}$, respectively).

Recent resequencing of the C3H genome has revealed many SNPs in the *Hcs7* region. Data compiled by the Mouse Genome Database (MGD) at the Mouse Genome Informatics website (Blake et al., 2011; URL: http://www.informatics.jax.org; 8/2011) show that the C3H alleles of eight genes in the *Hcs7* region encode amino acids that differ from B6. *Ifi202b* carries a Thr/Ser polymorphism. The most dramatic changes lie in *Mndal*, which carries Arg/Gly and Asp/Tyr polymorphisms. Other genes in the interval with non-synonomous SNPs include *Olfr433, Olfr419, Olfr220, Fmn2, Chml,* and *Exo1*.

4. Discussion

The Chromosome 1 liver cancer modifier *Hcs7* has been localized to a 3.3 Mb region on distal Chromosome 1. In DEN-treated mice, the C3H allele of *Hcs7* bred onto a resistant B6 background confers 3- to 7-fold greater tumor multiplicity, dominantly. Consistent with these results, the B6 allele of *Hcs7* bred onto a susceptible C3H background confers 4-fold resistance, recessively. Importantly, C3H alleles on Chromosome 1 including *Hcs7* confer susceptibility to spontaneous tumorigenesis that is comparable to the susceptibility of inbred C3H mice. The Chr 1 hepatocarcinogenesis susceptibility allele in C57BR/cdJ mice, *Hcif2*, does not appear to correspond to *Hcs7*. B6.BR mice congenic for BR alleles distal of 170 Mb are not more susceptible to liver tumorigenesis than B6 mice.

The initiation of tumors after DEN injection is thought to involve the proliferation of DEN-mutated hepatocytes in response to DEN-induced cell death, within 72 hours of injection (Maeda et al., 2005). We found that *Hcs7* affects neither apoptosis nor mitosis in this time period. We have previously shown that preneoplastic lesions in inbred C3H males grow faster than B6 lesions, and that this differential growth can be seen from prior to 16 weeks until at least 28 weeks of age (Hanigan et al., 1988). Here, we show that *Hcs7* accounts for part of this early growth advantage: the volume of preneoplastic lesions in the B6.C3H congenic lines 1R33 and 2R8 is greater than for B6 lesions by 16 weeks and remains so at 24 weeks. Our results indicate that *Hcs7* begins to affect cell proliferation after the acute reaction to DEN injection and likely tumor initiation, but before 16 weeks, to promote lesion growth.

The 175.35 to 178.64 Mb minimal interval carries 44 genes, of which 12 are related pyrin- and/or p200-domain-containing interferon-gamma response genes such as *Ifi202b*, *Aim2*, and *Mnda*. Two additional genes encode proteins that are known or likely to mediate immune responses (*Exo1* and the *Crp*-like gene 1810030J14Rik). This region also contains the cytoskeletal/cell polarity gene *Fmn2* that is overexpressed in B-cell leukemias, a renal cell cancer tumor suppressor (*Fh1*), four known or likely signaling protein genes, and 14 olfactory receptor genes.

Ifi202b is a strong candidate for the HCC modifier gene in the *Hcs7* locus. It was the only gene significantly differentially expressed on a genome-wide basis between B6.C3H^{Hcs7} congenics and B6 mice at 13 days of age (in the presence and absence of DEN) and in tumors from 32-week-old males. Importantly, CGH analysis indicates that the 5' end of *Ifi202b* is altered in the resistant B6.C3H congenic line 1R5, though the effect of this alteration on the protein or its expression still needs to be established. Finally, *Ifi202b* lies in the minimal susceptibility region determined by congenic lines, between 175.35 and 178.64 Mb. An important test of its candidacy will be to determine whether the CBA inbred strain, closely related to C3H and similarly susceptible to liver tumors, also expresses high levels of *Ifi202b*.

When only genes in the minimal susceptibility region were evaluated for differential expression in tumors, *Aim2* was the only other significantly differentially expressed gene. Aim2 is an interferon-regulated pro-apoptotic protein that is closely related to, can hetero-dimerize with, and affects the expression of *Ifi202b* (Panchanathan et al., 2010; Choubey et al., 2010). Aim2 is a component of inflammasomes that sense cytoplasmic double-stranded DNA and activate caspase 1, which in turn activates IL1-β and induces inflammation (Fernandes-Alnemri et al., 2009; Bürckstümmer et al., 2009).

In transfected macrophage cell lines, *Ifi202b* overexpression reduces *Aim2* expression, and *Aim2* knock-down increases *Ifi202b* expression (Panchanathan et al., 2010). Similarly, *Aim2* null mice overexpress *Ifi202b* in spleen tissue. Absence of *Aim2* expression correlates with cell growth *in vitro* and with cell immortalization *in vitro*, and *Aim2* is inactivated in approximately half of colon cancers with microsatellite instability (Woerner et al., 2007; Choubey et al., 2010). Therefore, high levels of *Ifi202b* may prevent *Aim2* expression and induce growth. Both *Aim2* and *Ifi202b* were overexpressed in line 2R8 tumors relative to B6 tumors. This result may reflect a tumor-specific response, as 13-day-old line 2R8 mice did not express more *Aim2* than B6 mice.

The high expression of *Ifi202b* in line 2R8 mice relative to B6 might reflect promoter and/or enhancer polymorphisms in both *Aim2* and *Ifi202b*. The NZB allele of *Ifi202b* has been shown to be more active than the B6 allele, most likely due to a TATA-box-creating polymorphism in the NZB *Ifi202b* promoter (Choubey et al., 2010). Analysis of congenic lines suggests that B6-specific polymorphisms, conversely, enhance the expression of *Aim2*. Autoimmune-sensitive congenics carrying NZB alleles from 154.7 to ~194 Mb express high levels of *Ifi202b* mRNA and protein relative to B6. However, mice from a line that carries a smaller NZB congenic region between approximately 174.5 to 194 Mb – including the entire *Aim2* gene at 175.2 Mb and the entire *Ifi202b* gene at 175.9 Mb – are not predisposed to autoimmunity. These resistant congenic mice express more *Aim2* mRNA and protein and less *Ifi202b* mRNA and protein than the larger congenic. Indeed, levels of Ifi202b protein are similar to those of B6 mice (Panchanathan et al., 2010). The breakpoint between B6 and NZB alleles in this smaller congenic lies less than one megabase proximal of *Aim2*. The effect of B6 polymorphisms near the 5' end of the *Aim2* gene on *Aim2* expression and, indirectly, *Ifi202b* expression may also explain the resistance to hepatocarcinogenesis of line 2R9. Assessing *Aim2* and *Ifi202b* mRNA and protein levels in lines 2R8, 2R16, 2R9, and 2R7 would address this hypothesis.

Interferon-inducible p200 family members have been shown to interact functionally with p53, MyoD, and Rb and appear to regulate proliferation, differentiation, apoptosis, and senescence (Gariglio et al., 2011). Overexpression of *Ifi202b* in particular has been shown to promote cell survival, while lower levels of expression increase cell death (Roberts et al., 2009; Choubey et al., 2010). The expression of *Ifi202b* is upregulated by IL-6, which has been shown to be virtually required for hepatocarcinogenesis in DEN-treated mice, and *Ifi202b* can stimulate or inhibit the transcription of NFκB target genes depending on cell type (Naugler et al., 2007; Choubey et al., 2010). Given these many cell regulatory functions and its effect on *Aim2* expression, *Ifi202b* could promote lesion growth in multiple ways. Determining whether preneoplastic cells in lines carrying C3H alleles undergo less cell death, an increase in mitosis, or both, will be an important first step in assessing the oncogenicity of *Hcs7*.

Based on genome-wide analysis, no genes other than *Ifi202b* were significantly differentially expressed. However, nine genes were not represented on the expression array. These include several unlikely candidates (olfactory receptors and splicing RNAs that are found throughout the genome), as well as the pyrin/p200 proteins *BC094916*, *Pydc3*, and *Mndal*. The expression of these genes and the effect of amino acid changes in genes in the region will need to be evaluated. Finally, the test of *Ifi202b* as a candidate will require its over-expression (*e.g.*, from a transgene) in B6, or its disruption in C3H.

If Ifi202b is the molecule responsible for promoting hepatocarcinogenesis in mice carrying C3H *Hcs7* alleles, it would make a tempting target for pharmacological intervention. The *Hcs7* region corresponds to parts of human chromosome 1q22-23.1 and 1q43. Chromosome 1q, including these regions, is amplified in 58% to 86% of human hepatocellular carcinomas, independent of stage (Thorgeirsson and Grisham, 2002; Lau and Guan, 2005; Chochi et al., 2009; Zhang et al., 2010). When only parts of 1q are amplified, 1q22-23 is frequently affected. These observations imply that chromosome 1q alterations are an early event in hepatocarcinogenesis. Chromosome 1q is also frequently amplified in other liver cancers such as hepatoblastoma, cholangiocarcinoma, and fibrolamellar carcinoma, as well as cancers of other tissues such as the pancreas and the breast (Buendia, 2002; Climent et al., 2002; Birnbaum et al., 2011; Ward and Waxman, 2011). Humans have four homologs of *Ifi202b* in the 1q22-23.1 region, including an ortholog of *Aim2*, an ortholog of *Mnda*, *Ifi16* and *IfiX/Pyhin1* (www.ensembl.org; 9/2011; Gariglio et al., 2011). All of these genes have been implicated in growth suppression in cell lines and ectopic tumor models; however, *Ifi16* in particular is also associated *in vivo* with rapidly dividing epithelium and with undifferentiated tissue including fetal hepatocytes and hepatocytes following liver transplantation (Gariglio et al., 2011). In an analysis of gene expression in a human tumor carrying a 1q amplification, four chromosome 1 genes were overexpressed. Only two of these correspond to mouse Chromosome 1: *Mnda* and "interferon-gamma inducible gene" at 1q22 (*Ifi16* or *IfiX*; Niketeghad et al., 2001). All four *Ifi* p200 human genes express transcripts that encode both the pyrin cell death and p200 protein interaction domains (www.ensembl.org; 9/2011). Each also expresses transcripts that carry only one of the domains, and *Ifi16* in particular expresses transcripts that carry only the pyrin domain, only the p200 domain, or both. These many alternative transcripts suggest the possibility that one of them is orthologous to *Ifi202b*, which expresses only the p200 domain. An analysis of human tumors should reveal which p200 family transcripts they express. Transgenesis could then be used to test the oncogenicity of these selected transcripts.

C3H alleles from several regions of mouse Chromosome 1 conferred some significant susceptibility to B6 mice. Similarly, several regions on human chromosome 1 are amplified, sometimes simultaneously but separately, in human tumors (Lau and Guan, 2005; Chochi et al., 2009). It seems likely that multiple genes on Chromosome 1 in both mice and humans affect hepatocarcinogenesis.

5. Conclusion

We have mapped the mouse *Hcs7* liver cancer modifier to a 3.3 Mb region on distal Chromosome 1 that carries 44 genes, of which many are involved in responding to immune stimuli. This region corresponds to human chromosome 1q22-23.1, which is amplified in most hepatocellular carcinomas. *Ifi202b* is a strong candidate gene for the *Hcs7* modifier. Further study of the effect of its overexpression or disruption *in vivo* on short-term proliferation or apoptosis and long-term hepatocarcinogenesis will help establish whether its possible functional equivalent on human chromosome 1q is a worthy target for pharmacological intervention.

6. Acknowledgements

The authors would like to thank Drs. Henry Pitot and Ruth Sullivan for liver histopathology training and diagnoses. We thank Bradley Stewart (McArdle) for microarray processing and

the Lab Animal Resources technicians for animal care. We thank Christopher Oberley for reading the manuscript and Matt Gigot, Kim Luetkehoelter, Tonia Jorgenson, Manching Leung, Heather Sturm, Katie Halser, Carl Koschmann, Henri Kurniawan, Jessica Linzmeyer, Myrra Windau, Fatima Ashraf, Bridget Borg, and Eric Simpson for their contributions. This work was supported by grants from the U.S. National Institutes of Health (CA22484, CA96654, CA07175, CA09135, and CA14520).

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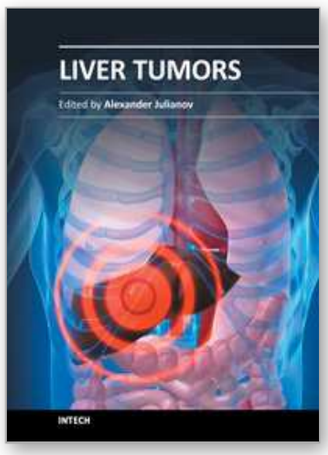

Liver Tumors

Edited by Prof. Alexander Julianov

ISBN 978-953-51-0036-2
Hard cover, 200 pages
Publisher InTech
Published online 03, February, 2012
Published in print edition February, 2012

This book is oriented towards clinicians and scientists in the field of the management of patients with liver tumors. As many unresolved problems regarding primary and metastatic liver cancer still await investigation, I hope this book can serve as a tiny step on a long way that we need to run on the battlefield of liver tumors.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Andrea Bilger, Elizabeth Poli, Andrew Schneider, Rebecca Baus and Norman Drinkwater (2012). The Hcs7 Mouse Liver Cancer Modifier Maps to a 3.3 Mb Region Carrying the Strong Candidate Ifi202b, Liver Tumors, Prof. Alexander Julianov (Ed.), ISBN: 978-953-51-0036-2, InTech, Available from: http://www.intechopen.com/books/liver-tumors/the-hcs7-mouse-liver-cancer-modifier-maps-to-a-3-3-mb-region-carrying-the-strong-candidate-ifi202b

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