Published in final edited form as:
Cancer Res. 2012 November 15; 72(22): 6002–6012. doi:10.1158/0008-5472.CAN-12-0748.

Rat *Mcs1b* is concordant to the genome wide association identified breast cancer risk locus at human *5q11.2* and *Mier3* is a candidate cancer susceptibility gene

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Abstract

Low-penetrance alleles associated with breast cancer risk have been identified in population-based studies. Most risk loci contain either no or multiple potential candidate genes. Rat *mammary carcinoma susceptibility 1b* (*Mcs1b*) is a quantitative trait locus (QTL) on *RN02* that confers decreased susceptibility when Copenhagen (COP) resistant alleles are introgressed into a Wistar Furth (WF) susceptible genome. Five WF.COP congenic lines containing COP *RN02* segments were compared. One line developed an average of 3.4 ± 2.0 and 5.5 ± 3.6 mammary carcinomas *per* rat ± SD when females were *Mcs1b* resistant homozygous and *Mcs1b* heterozygous, respectively. These phenotypes were significantly different from susceptible genotype littermates (7.8 ± 3.1 mean mammary carcinomas *per* rat ± SD, P = 0.0001 and P = 0.0413, respectively). All other congenic lines tested were susceptible. Thus, *Mcs1b* was narrowed to 1.8 Mb of *RN02* between genetic markers *ENSRNOSNP2740854* and *g2UL2-27*. Mammary-gland-graft carcinoma-susceptibility assays were used to determine that donor (P = 0.0019), but not recipient *Mcs1b* genotype (P = 0.9381), was associated with ectopic mammary carcinoma outcome. Rat *Mcs1b* contains sequence orthologous to human *5q11.2,* a breast cancer susceptibility locus identified in multiple genome-wide association studies. Human/rat *MAP3K1/Map3k1* and *MIER3/Mier3* are within these orthologous segments. We identified *Mier3* as a candidate *Mcs1b* gene based on 4.5-fold higher mammary gland levels of *Mier3* transcripts in susceptible compared to *Mcs1b* resistant females. These data suggest that the human *5q11.2* breast cancer risk allele marked by *rs889312* is mammary-gland autonomous, and *MIER3* is a candidate breast cancer susceptibility gene.

Keywords

breast cancer susceptibility; rat *Mcs1b*; rat mammary cancer; complex disease genetics; comparative genetics

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Potential conflicts of interest: None.

Introduction

Low-penetrance breast cancer susceptibility alleles have been identified using human genome-targeted and genome-wide association study (GWAS) designs [1–13]. Apart from identifying complex disease risk-associated genetic variation, human studies alone are limited in pinpointing candidate genes and functional characterization of susceptibility associated loci. Comparative genetics based on experimental organisms such as *Rattus norvegicus* (laboratory or Norway rat) recapitulate the complex genetics, and may complement and enhance human studies of breast cancer risk and prevention [14, 15].

The laboratory rat provides a good model of human breast cancer. Rat mammary carcinomas closely resemble human breast carcinomas in histopathology, hormonal responsiveness, and potential environmental etiologies [16–22]. Rats develop spontaneous, carcinogen, and oncogene induced mammary carcinomas [23]. Several rat strains representing different spectrums of genetic variation in susceptibility to mammary carcinogenesis exist [24–29]. Genotypic differences in susceptibility are not necessarily due to carcinogen-induced differences in metabolism or DNA damage [26, 30].

Multiple rat *mammary carcinoma susceptibility* (*Mcs*) quantitative trait loci (QTLs) have been identified [28, 29, 31]. Rat *Mcs* QTLs that are concordant to human breast cancer risk alleles may be used to identify genes and mechanisms controlling breast cancer susceptibility in women. Comparative genetic approaches have been used to identify *MCS5A1* and *MCS5A2*, which are common noncoding breast cancer risk alleles on human Chr *9* [1]. Human *MCS5A1* has been confirmed to associate with breast cancer risk in additional population-based studies [9]. Another rat QTL, *Mcs6,* was mapped to an orthologous region of human *Chr 12*, which potentially associates with breast cancer risk [32]. Rat genetics will continue to play an important role in elucidating breast cancer risk alleles, candidate genes, and molecular mechanisms.

Rat *Mcs1b* was initially mapped to a 16 cM region of rat Chr *2* [33]. Positional mapping was completed in WF.COP congenics by introgression of a segment of resistant Copenhagen (COP) Chr *2* into a susceptible Wistar-Furth (WF) genetic background. *Mcs1b* resistant alleles decrease mammary carcinoma multiplicity compared to susceptible WF alleles. We have mapped rat *Mcs1b* to a shorter genomic interval, and found that it contains the rat ortholog to a GWAS identified human breast cancer risk associated allele at human Chr *5q11.2* that is marked by SNP *rs889312* [2]. This locus has been confirmed to associate with human breast cancer in different human populations and tumor sub-types [34–39]. We have further utilized rat *Mcs1b* congenic lines to investigate functional aspects of these alleles and identify *Mier3* as a strong mammary cancer susceptibility gene.

Materials and Methods

Animals and Phenotyping

Congenic rat lines were maintained in an AAALAC-approved facility on a 12 h light/dark cycle and provided LabDiet 5001 Rodent Diet (PMI® Nutrition International) and water *ad libitum.* All animal protocols were approved by the University of Louisville Animal Care and Use Committee. Congenics are defined as genetic lines that carry defined COP alleles introgressed into the inbred WF/NHsd (Harlan) genome. Information for genetic markers defining ends of COP alleles carried by each congenic line T, N3, F3, W2, U2, and I4 is available at the UCSC Genome Browser (www.genome.ucsc.edu), Rat Genome Database (http://rgd.mcw.edu/), NLM NCBI, or Supplementary Table S1. At 50–55 days of age, 7,12-dimethylbenzanthracene (DMBA, 20 mg/mL sesame oil) was given by a single oral gavage (65 mg DMBA/kg body mass). Mammary carcinoma susceptibility phenotypes were

determined by counting (multiplicity) mammary carcinomas ≥ 3×3 mm^2 that developed 15 weeks after carcinogen [33].

Mammary Gland Grafting

Mammary gland grafting experiments used WF.COP line N3 females at N16F5, N16F6, and N16F7 generations to supply *Mcs1b* resistant alleles. At the N15 generation, line N3 was backcrossed to the inbred WF/NHsd strain and re-fixed at the N16 generation for the $Mcs1b^{COP}$ allele it carries. Using congenics, opposed to F_1 rats, provided rats homozygous at *Mcs1b*, which provided higher penetrance without influence from other COP resistance alleles. Both abdominal and adjacent inguinal mammary glands with lymph nodes removed were excised from 30–35 day old donor females. Mammary tissue was finely minced in a Petri dish on ice, divided into four equal volumes, and placed onto the interscapular white fat pad of 30–35 day old recipients (1 donor *per* 4 recipients). DMBA was administered as before. Interscapular fat pads were evaluated at 15 weeks following DMBA for graft site tumor development. Ectopic mammary tumors were histologically verified to be carcinomas. Interscapular fat pad tissue was whole mounted and stained with aluminum carmine to determine if an ectopic mammary gland had developed.

Resequencing

Total RNA samples, extracted using TriReagent (Molecular Research Center) and standard chloroform/isopropanol precipitation, were from WF/NHsd and WF.COP lines N3 and T. TURBO DNase (Life Technologies) was used to reduce DNA contamination and cDNA was made using Superscript III reverse transcriptase (Life Technologies). In some instances sequences were not attainable from cDNA. To obtain these sequences genomic DNA, extracted from frozen spleen or liver tissues using standard phenol-chloroform/isopropanol precipitation, was used. Ampliphied samples were cleaned with the QIAquick PCR Purification Kit (Qiagen) prior to using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Life Technologies). Sequencing reactions were purified with Agencourt CleanSeq magnetic beads (Beckman Coulter) and analyzed by the University of Louisville Center for Genetics & Molecular Medicine DNA Core using an ABI PRISM 7700 Sequence Detection System (Life Technologies). Primer sequences for amplifying and sequencing *Mcs1b* ORFs and 3′-UTRs are in Supplementary Table S2. Nucleic acid sequences were submitted to NCBI/GenBank and assigned accession numbers JQ013728 thru JQ013737.

Quantitative PCR

Total RNA was isolated with TRI-Reagent (Molecular Research Center) from flash-frozen and homogenized tissues. To reduce possible solvent and DNA contamination RNA samples were further processed by a 1/10 v/v 3M sodium acetate and 2.5x v/v 100% ethanol wash on ice for 10 minutes followed by 80% ethanol wash followed by Turbo DNAse (Life Technologies). Total RNA quantity and quality were measured with a Nanodrop 1000 (Fisher Scientific) and a Bioanalyzer with RNA 6000 NanoChips (Agilent). Reverse transcription reactions (20 μl f.v.) that contained 1μg total RNA, 0.5x RNAsecure, 5μM random hexamers, 25ng/μL oligo(dT_{18}), and 0.5 mM dNTPs were incubated 5 minutes at 65° C prior to adding 1× first strand buffer, 100mM DTT, and 1μL Superscript III (Life Technologies). Reactions were incubated 5 m at 25° C, 1 h at 50° C, and 15 m at 70° C. Quantitative PCR was performed as previously published [1] using TaqMan MGB probes (Life Technologies) and primers in Supplementary Table S3. Except, 60 nM each *Rplp2* primer and 120 nM VIC-labeled *Rplp2* probe were used as an endogenous control. Fluorescence values were measured using SDS v2.3 software (Life Technologies).

Plasmid Construction and MIER3 Expression

Homo sapiens MIER3 ORF (NCBI/GenBank ref|NM_152622.3) from pooled human breast total RNA (#AM6952, Life Technologies) was cloned into pEGFP-C1 vector at EcoRI and KpnI sites. Primer sequences for cDNA amplification were 5′-CGGAATTCTATGGCGGAGGCTTCTTTTGGAAGT and 5′-CGGGGTACCCTCAGAGTGTAGGGCCGCGTGC. MDA-MB-231 (#HTB-26) and T47D (#HTB-133) cell lines were purchased from American Type Culture Collection (ATCC) in May 2011, cultured, respectively, in DMEM with 10% FBS and RPMI 1640 with 10% FBS and 0.2 U/mL bovine insulin, and cyropreserved after one passage for future use. Cell authentication was guaranteed by ATCC and morphology was confirmed under a phase-contrast light microscope. Cells (6.25×10^5 MDA-MB-231/well or 2×10^5 T47D/well) in 6-well plates were transiently transfected with 2.5 μg of pEGFP-hMIER3 or pEGFP-C1 plasmids using Lipofectamine LTX/Plus (Life Technologies). Samples were visualized at 24 h using a phase-contrast or confocal (Olympus IX51 40x objective) microscope. Cells were grown on cover slips in chambers (Lab-Tek #177445), washed with PBS, fixed for ten minutes with 10% paraformaldehyde, and washed again with PBS prior to DAPI (2 ng/mL PBS) staining for five minutes. Cover slips were mounted on microscope slides using fluorescent mounting solution (DAKO). Fluorescent images were captured using cellSens Dimension software (Olympus).

Genomics and Statistical Analysis

Genome assemblies used were *Homo sapiens* version GRCh37/hg19 and *Rattus norvegicus* version 3.4/rn4. Mammary carcinoma multiplicity phenotypes were compared by nonparametric Mann Whitney tests. Results from mammary gland grafting experiments were analyzed using logistic regression. Donor and recipient genotypes were incorporated as dependent variables. In independent models, graft site tumor outcome and grafting ability were used as independent variables. Quantitative PCR (QPCR) data were analyzed using ANOVAs with $\log_2$ (*Target* quantity/*Rplp2* quantity) as the dependent variable. Independent variables for comparing mammary gland transcript levels were *Mcs1b* genotype and DMBA exposure. *Mcs1b* genotype and tissue source were independent variables for mammary carcinoma and non-diseased mammary tissue QPCRs. Fisher's PLSD tests were used to compare groups following a significant F-test ($\alpha \leq 0.05$). Statview software (SAS Institute) was used.

Results

Fine-Mapping *Mcs1b* using WF.COP Congenics

Subsequent comparative genetics work is reduced if QTLs are mapped to short syntenic intervals. Five congenic lines that contained different resistant COP rat Chr *2* segments of the *Mcs1b* candidate region from *D2Uwm17:D2Rat200* (Chr*2*:32051320-48762858) on a susceptible WF genetic background were tested to narrow *Mcs1b* (Fig. 1). Mammary carcinoma susceptibility phenotypes were determined using tumor multiplicity at 15 weeks following DMBA induction of mammary carcinogenesis. A shorter segment of COP Chr *2* that was contained in congenic line N3 conferred a decreased *Mcs1b* phenotype similar to line T (Table 1). Females of line N3 that were *Mcs1b* resistant homozygous or heterozygous developed, respectively, 56% and 30% less mammary carcinomas *per* rat than *Mcs1b* susceptible homozygous (WF/WF) female littermates from line N3. Congenic lines F3, W2, U2, and I4 each contained different COP *Chr 2* segments (Fig. 1). All these lines had Mcs phenotypes similar to littermates with susceptible WF genotypes (Table 1). Comparison of microsatellite DNA and published rat SNPs located in the 0.66 Mb of genomic sequence between the distal and proximal ends of lines N3 and I4 yielded no genetic variation between resistant COP and susceptible WF alleles (Supplementary Table S4). Therefore, we

were unable to define a precise distal end to *Mcs1b*. When considered together, our congenic line data delimit *Mcs1b* to a ~1.8 Mb of rat Chr *2* that spans from SNP *ENSRNOSNP2740854* to microsatellite *g2UL2-27*, which corresponds to rat Chr*2*:42364155-44195382 (Fig. 1).

Mcs1b Mammary-Gland-Graft Carcinoma Susceptibility

To determine if the *Mcs1b* resistant allele acted to reduce mammary cancer susceptibility in a mammary gland autonomous manner, we subjected animals with ectopically transplanted mammary-gland tissue to DMBA-induced mammary carcinoma susceptibility assays. Females used in mammary grafting assays had a WF genetic background and either *Mcs1b* resistant or susceptible WF alleles. We expected these animals to have compatible immune systems; and thus, not reject reciprocal mammary gland grafts. To test this expectation, it was determined empirically that recipients did not reject mammary tissue grafts from donors of different *Mcs1b* genotypes. The respective total number of recipients (r) and ectopic mammary-graft positive recipient (+r) females in susceptible (S) and resistant (R) reciprocal donor:recipient transplant groups are reported in Table 2. There were no statistically significant associations between mammary tissue grafting ability and donor or recipient genotype.

Data from ectopic mammary gland graft positive recipients were analyzed to test for associations of donor and recipient genotypes with carcinoma development at the ectopic mammary gland site. Donor mammary tissue from *Mcs1b* resistant (R) females, when grafted into interscapular white fat pads of either R or susceptible (S) recipients resulted in fewer females developing ectopic mammary carcinomas compared to recipients of either genotype that received mammary tissue from S genotype donors (Fig. 2). Donor, but not recipient genotype, was significantly associated with graft-site mammary carcinoma outcome (Table 2). These results indicated that *Mcs1b* conferred resistance is mammary gland autonomous. This also suggested that work to characterize this locus should initially focus on mammary tissue.

Resequencing *Mcs1b* Potential Candidate Open Reading Frames

As shown in Fig. 3, rat *Mcs1b* was found to contain thirteen potential candidate gene transcripts as well as sequence orthologous to human *5q11.2*, a GWAS-identified breast cancer risk associated allele marked by SNP *rs889312* [2]. To prioritize potential candidates we resequenced conserved protein coding open reading frames (ORFs) that were within the 1.8 Mb interval that delimited *Mcs1b,* and based on RT-PCR gel electrophoresis were expressed in mammary glands of susceptible WF and *Mcs1b* resistant females (lines N3 and T). Transcripts from *Gpbp1, Map3k1, Mier3, Ankrd55, Il6st, Il31ra, Ddx4, Slc38a9,* and *Ppap2a* genes were detected in mammary gland total RNA pools from each genotype by RT-PCR. No genetic variants were identified between susceptible WF and *Mcs1b* resistant genotype ORFs or 3′ UTRs for these transcripts. Nucleotide sequences were submitted to NLM-NCBI.

Four of the *Mcs1b* potential candidate genes were predicted transcripts (gray bars in Fig. 3). Rat *Actbl2* (A in Fig. 3) is a psuedogene located outside rat genomic sequence orthologous to the human *5q11.2* haplotype block that associates with breast cancer risk. Predicted transcript *ENSRNOG00000013098* (B in Fig. 3) was listed on the Ensembl genome browser [40]. We found no evidence by RT-PCR of a transcript from *Actbl2* or *ENSRNOG00000013098* in total RNA samples from multiple susceptible and *Mcs1b* resistant mammary glands or in rat mixed tissue total-RNA samples that included embryo, brain, testes, ovary, thymus, spleen, and liver. Since cDNA was not attainable and *Actbl2*

was predicted to be a single-exon we sequenced genomic DNA spanning this predicted psuedogene and found no sequence differences between WF and COP alleles.

A rat orthologous transcript (C in Fig. 3) to predicted *C5ORF35* was not present in any total RNA samples tested from various rat tissues. We successfully amplified *C5ORF35* from human thymus, spleen, and ovary, but not human breast tissue cDNA (Supplementary Fig. S1). However, in an Oncomine [41] search we found that other groups have reported detection of *C5ORF35* in human breast carcinoma and non-diseased breast tissue. We noted that the annotated 5′- and 3′-UTRs of human *C5ORF35* are poorly conserved between humans and rodents (Fig. S1); therefore, we concluded that *C5ORF35* is a human, but not a rat transcript.

A predicted small nuclear RNA at rat Chr *2:43765811-43765918:1* named *U6* or *ENSRNOG00000034909* (D in Fig. 3) is estimated to be 108 bp on the forward strand. We noted that *ENSRNOG00000034909* sequence aligned to multiple regions of the rat genome using both NCBI/BLAST and UCSC/BLAT [42, 43]. Thus, because of the highly repetitive nature of the sequence we were unable to design specific probes to determine if this predicted single exon gene was transcribed from rat *Mcs1b.*

Mcs1b Potential Candidate Gene Transcript Levels

Rat *Mcs1b* did not contain any protein coding genetic variation between *Mcs1b* susceptible and resistant alleles; therefore, rat *Mcs1b* may contain variation in one or more non-protein-coding regulatory elements that differentially control gene expression between mammary cancer susceptible and resistant genotypes. To test this hypothesis we measured mammary gland transcript levels of genes located at *Mcs1b* in 12-week old virgin female rats that were exposed to DMBA at 50–55 d to induce mammary carcinogenesis and age matched controls without DMBA. We focused on mammary gland transcript levels due to the mammary gland autonomous nature of *Mcs1b.* Twelve-week old animals were used because this age is after acute DMBA-toxicity and before frank mammary carcinomas are detectable.

Potential candidate gene transcript levels between *Mcs1b* genotype and DMBA exposure were analyzed by two-way ANOVA (Table 3). Effect of *Mcs1b* genotype was statistically significant for *Gpbp1*, *Mier3*, *Map3k1*, and *Il6st.* There was a significant effect of DMBA exposure on *Map3k1* transcript levels. The interaction between *Mcs1b* genotype and DMBA exposure approached statistical significance for *Map3k1.* When mammary cancer susceptible and *Mcs1b* resistant genotypes were compared by exposure (with DMBA or without) mammary gland transcript levels were significantly different between susceptible and *Mcs1b* resistant females that were not carcinogen-induced for *Gpbp1, Mier3,* and *Map3k1.* Transcript levels of *Gpbp1* and *Map3k1* were not different between genotypes when DMBA-exposed females were evaluated. Significant expression differences between susceptible and *Mcs1b* resistant genotypes were sustained only for *Mier3* when females given DMBA were compared between genotypes. We did not observe statistically significant differences in transcript levels of *Ankrd55, Il31ra, Ddx4, Slc38a9,* or *Ppap2a* between susceptible and *Mcs1b* resistant genotype mammary glands with DMBA or without.

Mammary gland transcript levels were lower in *Mcs1b* resistant genotype females for all genes with a significant difference between genotypes. *Mier3* mean transcript levels were approximately 4.5-fold lower in *Mcs1b* resistant compared to susceptible genotype mammary glands whether animals were exposed to DMBA or not (Table 3). Thus, exposure to mammary carcinogen had no appreciable effect on *Mier3* differences between susceptible and *Mcs1b* resistant genotype females. No significant differences in *Mier3* transcript levels were detected between *Mcs1b* resistant and susceptible genotypes in spleen, thymus, ovary,

or brain tissues (Supplemental Fig. S2). This suggests that *Mier3* transcript level differences between *Mcs1b* alleles may be specific to mammary gland tissue.

A loss in statistical significance between DMBA-exposed susceptible and *Mcs1b* resistant females for *Map3k1* was due to a statistically significant (P = 0.0003) increase in mean level of *Map3k1* in the *Mcs1b* resistant genotype females with DMBA compared to age-matched controls of the same genotype without DMBA (Table 3). *Map3k1* levels were not different (P = 0.2038) between susceptible WF mammary glands with DMBA or without.

Effect of *Mcs1b* Genotype on Body Weight

Travis *et al.* detected a significant association between human breast cancer risk associated SNP *rs889312* and stature in women [44]. To determine if rat *Mcs1b* might also exhibit pleiotropy we analyzed rat body weight, which is information we routinely collect and relevant because body weight is genetically correlated to stature in humans [45]. Significant effects of *Mcs1b* genotype (P < 0.0001) and DMBA exposure (P = 0.0014) on body weight at 12 weeks of age were detected (Fig. 4A). The interaction between *Mcs1b* genotype and DMBA exposure was also significant (P = 0.0004). Females with the *Mcs1b* resistant genotype had mean ± SD body weights of 200 ± 11 grams with DMBA (n = 47) and 201 ± 7.7 grams without (n = 33), which were not significantly different (P = 0.7880). Comparatively, mammary cancer susceptible females had higher (P < 0.0001) mean ± SD body weight at 192 ± 11 grams with DMBA (n = 45) than unexposed susceptible females (n=34) who had a mean ± SD body weight of 180 ± 12 grams.

Higher *Mier3* Transcript Levels in Mammary Carcinomas

Transcript levels of *Mier3, Il6st, Gpbp1, and Map3k1* in DMBA-induced mammary carcinomas that developed in susceptible (n=28) and *Mcs1b* resistant genotype (n=25) mammary glands were measured by QPCR to determine if there was an effect of *Mcs1b* genotype on levels of any of these transcripts in mammary carcinoma tissue. These genes were evaluated because significant effects of *Mcs1b* genotype on mammary gland transcript levels of these genes were detected (Table 3). Further, *Il6st* was included because it had been reported to be higher in rat mammary carcinoma compared to mammary gland tissues [46]. We collected total RNA from DMBA-induced mammary carcinomas (n= 1 or 2 *per* rat) and adjacent non-diseased mammary gland tissue from 21–23 week old females (n= 6 *per* genotype). There were no statistically significant differences in mammary carcinoma transcript levels between *Mcs1b* genotypes for any of the four genes tested. However, as shown in Fig. 4B, *Mier3* transcript levels were significantly higher (1.8-fold) in mammary carcinomas compared to non-diseased mammary tissue. We also observed that *Il6st* was potentially different between mammary carcinomas and non-diseased mammary glands; however, this comparison did not meet our statistical significance criterion (Fig. 4B).

Oncomine [41] was used to query The Cancer Genome Atlas (cancergenome.nih.gov) gene expression database to find that levels of human *MIER3* were, respectively, 1.33 and 1.20 fold higher in invasive ductal (n = 392) and invasive lobular (n = 36) breast carcinoma samples compared to pathologically normal breast tissues (n = 61) ($P = 2.8 \times 10^{-13}$, ductal; $P = 6.3 \times 10^{-4}$,lobular; t-tests, Fig. 4C). Thus, both human/rat *MIER3/Mier3* levels are higher in breast/mammary carcinoma compared to non-diseased breast/mammary tissues.

Localization of Human MIER3 Protein to Nuclei

MIER3 encodes an uncharacterized member of the mesoderm induction early response family of proteins. The gene product of family member *MIER1* is a transcription factor targeted to the nucleus [47]. Therefore, we determined if *MIER3* may also encode a transcription factor targeted to the nucleus. We cloned a human MIER3 ORF into an

enhanced green fluorescent protein (eGFP) expression vector and transiently transfected this expression vector into MDA-MB-231 and T47D breast cancer cell lines. *In vitro* expression of MIER3 linked to eGFP resulted in green fluorescence in distinct *foci* compared to eGFP alone (Supplementary Fig. S3). We confirmed that *foci* were nuclei by determining that MIER3-eGFP co-localized with DAPI-staining in both cell lines (Fig. 5).

Discussion

Rat mammary carcinoma susceptibility, like human breast cancer risk, is complex as both are controlled by multiple susceptibility alleles and environmental factors. We have mapped rat *Mcs1b* to a 1.8 Mb region of rat chromosome *2* using multiple congenic lines. We found that rat *Mcs1b* is highly relevant to human breast cancer susceptibility as it contains genomic sequence orthologous to a low-penetrance breast cancer risk allele at human chromosome *5q11.2.* This human susceptibility allele was first reported by Easton *et al.* in the first population-based breast cancer risk GWAS [2]. Human *5q11.2* has been confirmed to strongly associate with breast cancer risk in multiple independent studies of European- and Asian-descent populations [34–39]. This is the first report of a rodent complex disease susceptibility QTL with a GWAS-identified concordant human ortholog that had a probability of association below a stringent significance level of $P \leq 10^{-7}$, which is widely deemed to be required for genome-wide studies.

An experimental organism with a segregating concordant susceptibility allele implies that functional genetic studies may translate directly to human biology and disease. For example, Gould and colleagues reported that rat *Mcs5a*, a Wistar Kyoto (WKY) strain resistance QTL that is concordant to human *MCS5A*, acted in a non-mammary cell-autonomous manner that involves immune cells [48]. Here, we used rat genetic lines to show that *Mcs1b* controls mammary cancer susceptibility by an undetermined mechanism that is autonomous to mammary gland tissue. While our result is in agreement with previous work that concluded a majority, but not all, of the COP rat strain resistance to mammary cancer is mammary gland autonomous [49]; it further highlights that the WKY and COP strains may achieve mammary carcinoma resistance thru different genetically determined cellular and molecular mechanisms. Mechanisms that are likely genetically determined in humans.

Most common genetic variation associated with human complex disease susceptibility appears to be located in non-protein-coding DNA. Since we found no genetic variation between susceptible and resistant allele *Mcs1b* ORFs, we conclude that *Mcs1b* is likely a noncoding gene regulatory element(s), such as a transcription factor binding site or noncoding RNA. This would be similar to the hypothesized identity of the human *5q11.2* breast cancer risk associated element. Human polymorphisms that are contained in public databases and highly correlated with human *5q11.2* breast cancer risk associated SNP *rs889312* are in non-protein-coding DNA. There are no known noncoding RNAs in either the human or rat ortholog; therefore, another type of gene regulatory element is likely responsible for or associated to susceptibility differences.

Our studies suggest that *MIER3* is a strong candidate breast cancer susceptibility gene at human *5q11.2.* We have identified *Mier3* as a strong *Mcs1b* candidate gene in this study based on different *Mier3* mammary gland transcript levels between susceptible and *Mcs1b* resistant genotypes. Lower levels of *Mier3* in *Mcs1b* resistant genotype females were genetically determined and not dependent on the induction of mammary carcinogenesis by DMBA. We also found *Mier3* levels to be significantly lower in non-diseased rat mammary tissue compared to mammary carcinoma. Further, we queried The Cancer Genome Atlas gene expression database and noted that human *MIER3* levels were higher in both ductal and lobular breast carcinomas compared to breast tissue.

MIER3 or *mesoderm induction early response 1, family member* 3 (GenBank ref| NM_152622) is an uncharacterized gene. We determined that MIER3 localized to the nucleus. Human and rat MIER3/Mier3 (GenBank ref| NP_689835.3 and NP_001161472.1) gene products share 93% amino acid sequence identity, and human MIER3 and MIER1 (GenBank ref|NP_001071172.1) have 54% identical amino acids based on BLAST [42]. MIER1 physically interacts with estrogen receptor alpha, Sp1, and Creb-binding protein [50–52]. MIER1 contains one, while MIER3 has two conserved LXXLL sequences, which is a motif that facilitates nuclear hormone receptor interactions [53]. A potential functional difference between MIER1 and MIER3 may be that a difference in the number of LXXLL motifs between them results in physical interactions with different nuclear hormone receptors [54].

In addition to *MIER3, MAP3K1* and *C5ORF35* reside within the human *5q11.2* haplotype block that associates with breast cancer risk. Even though there are no published studies in support, *MAP3K1* is often considered the candidate breast cancer susceptibility gene at *5q11.2* due to its location within the breast cancer risk associated haplotype block and known function as a serine/threonine kinase. In our rat studies, *Map3k1* was differentially expressed between susceptible and *Mcs1b* resistant congenic rats that had not been induced to undergo mammary carcinogenesis; however, mammary glands that had been exposed to mammary carcinogen did not show a difference in *Map3k1* levels between *Mc1b* alleles. An interesting result in our study with respect to *Map3k1*, which may have important implications for human studies of potential genotype-environment interactions, is exposure to mammary carcinogen resulted in increased mammary gland *Map3k1* levels for the *Mcs1b* resistant, but not the susceptible genotype. We found no evidence of a rat orthologous transcript to human *C5ORF35* in multiple rat tissues. Further, exonic elements of *C5ORF35* have not been conserved in the rat. Therefore, we conclude that *MAP3K1* and *C5ORF35* are not as likely as *MIER3* to be breast cancer susceptibility genes.

We noted that both rat *Mcs1b* and human *5q11.2* exhibit pleiotropy. Travis *et al.* reported that carriers of the increased risk allele at human *5q11.2* were significantly shorter in height than non-carriers [44]. In our study, high risk female rats had lower body weight than *Mcs1b* resistant females. We noted on the Rat Genome Database that there is a predicted rat body weight QTL named *Bw1* that overlaps *Mcs1b* and is associated with mesenteric body fat amount [55]. Both human and rat study results are counter intuitive as one might expect taller women and heavier rats to be at greater cancer risk. Thus, it is important to note that, as expected with low-penetrance alleles, the quantitative difference between the means for each human genotype were subtle with overlapping distributions. Mean height difference was 7 mm between non-carriers and carriers of the increased risk allele. In our study, we analyzed only body weight, and not specific components of body weight, such as bone density or fat amounts. Thus, better descriptive traits would likely be more informative. It is notable that the pleitropic effects of these alleles opens the possibility that other experimental organisms, approaches, and study designs without focus on breast or mammary cancer may be useful to functionally characterize breast cancer risk associated genetic variation at *5q11.2*.

In conclusion, rat *Mcs1b* is mammary gland autonomous allele and a non-protein-coding genetic element that is orthologous to the GWAS-identified human *5q11.2* breast cancer susceptibility locus. We propose that *MIER3* is a strong candidate breast cancer susceptibility gene.

Supplementary Material

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Acknowledgments

NIH grants R01CA137052 (DJS), P30ES014443 (DJS), T32ES011564 (ADD), and T35ES014559-06 (MDV); and Competitive Enhancement and Intramural Research Incentive Grants from the University of Louisville Office of the Executive Vice President for Research (DJS).

The authors thank Jason Willer, Jane Williams, Thomas Ray, Kendall Prater, and Deepa Patel for contributing technical assistance. This work was supported by National Institutes of Health grants CA137052 and ES014443, and partially supported by Competitive Enhancement and Intramural Research Incentive Grants from the University of Louisville Office of the Executive Vice President for Research. ADD and MDV were, respectively, supported by National Institutes of Health Training Grants T32ES011564 and T35ES014559-06.

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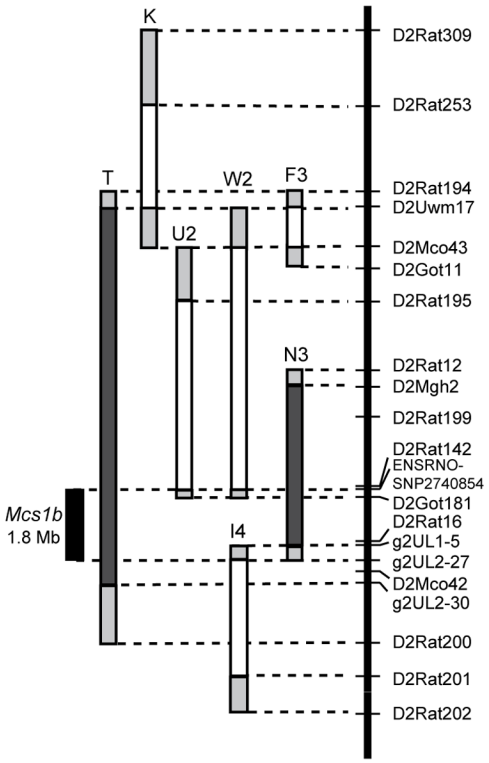

Figure 1. Rat Chr *2* map of WF.COP lines delimiting *Mcs1b* to 1.8 Mb

Markers used to genotype WF.COP congenics are listed in relative positions on the y-axis. Lines are labeled with letter-number combinations and designated with filled dark-gray bars to indicate *Mcs1b* resistant alleles. Lines that are drawn with unfilled bars represent COP intervals incapable of conferring decreased susceptibility or resistance to mammary carcinoma development. The filled light-gray bars at ends of each congenic segment are intervals of unknown genotype. Lines T and K were published previously [33].

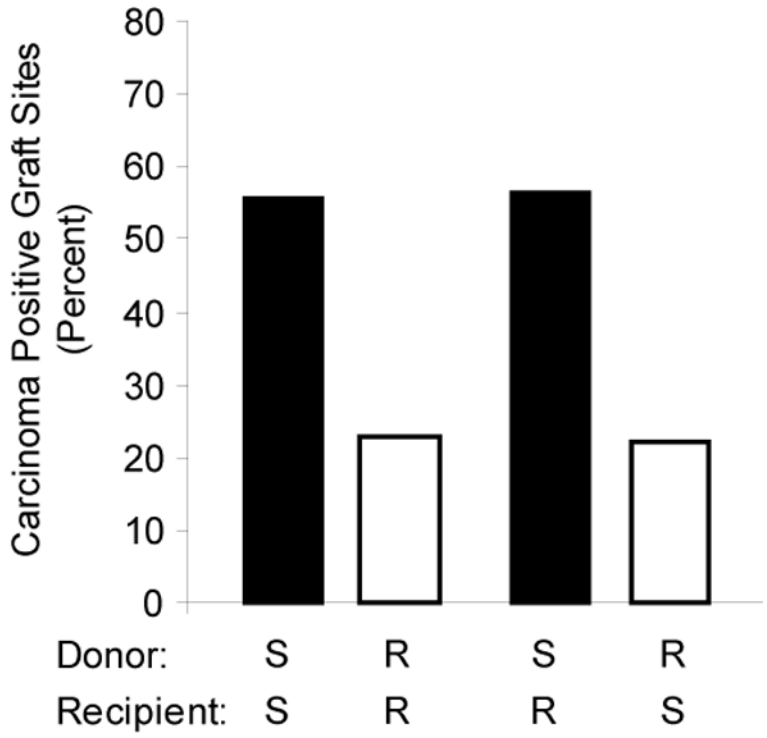

Figure 2. Rat *Mcs1b* is mammary gland autonomous

Percentage of mammary gland graft positive recipients that developed ectopic mammary gland carcinomas are shown for each susceptible (S) and *Mcs1b* resistant (R) donor:recipient group. Groups with a S donor are shown as filled bars, and groups with a R donor are shown as unfilled bars. The total number of mammary gland graft positive recipients that were evaluated for tumor outcome in each group were, respectively, 27, 22, 23, and 18 for S:S, R:R, S:R, and R:S.

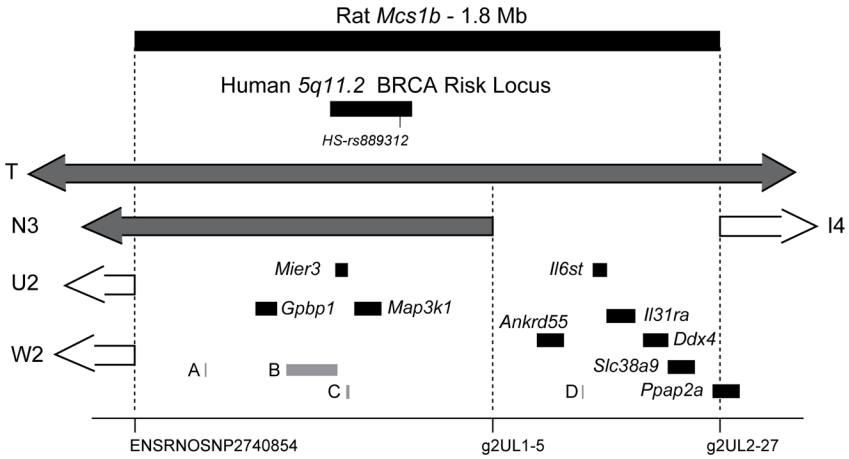

Figure 3. Rat *Mcs1b* contains the ortholog of GWAS identified human *5q11.2* breast cancer risk allele marked by SNP *rs889312* and multiple human/rat conserved transcripts

Genetic markers used to map *Mcs1b* to this region are marked on the x-axis. Ends of relevant congenic lines are included for orientation. Potential candidate breast cancer susceptibility gene transcripts mapping to rat *Mcs1b* are shown as filled black bars that represent exonic and intronic DNA. Gene names are flanking 5′-UTRs of respective transcripts. Elements shown as gray bars and designated as A, B, C, or D correspond to predicted *Actbl2, ENSRNOG00000013098, C5orf35,* and *ENSRNOG00000034909*, respectively.

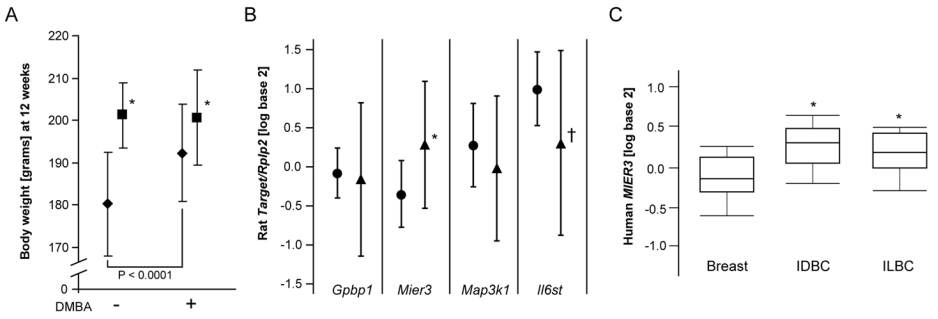

Figure 4. Rat *Mcs1b* resistant genotype is associated with higher body weight and rat/human *Mier3/MIER3* levels are higher in rat mammary and human breast carcinomas

Panel A: Lower body weight at 12 weeks of age was observed in mammary carcinoma susceptible (diamonds) compared to *Mcs1b* resistant females (squares) with DMBA and without ($P < 0.0001$ and $P = 0.0007$, respectively). Body weight was significantly higher in susceptible females that received DMBA compared to without ($P < 0.0001$). Panel B: Rat *Mier3* transcript levels were significantly higher in DMBA-induced mammary carcinomas (triangles) compared to non-diseased mammary gland tissue (circles, $P = 0.0120$). Mean ± SD are graphed for each variable. Panel C: Human *MIER3* was significantly higher in breast carcinomas compared to pathologically normal breast tissues. Oncomine (www.oncomine.org, reference 41 of main text) was used to query The Cancer Genome Atlas (cancergenome.nih.gov) gene expression database. Shown are box plots of $\log_2$ median centered *MIER3* transcript levels for invasive ductal breast carcinomas (IDBC, n=392) and invasive lobular breast carcinomas (ILBC, n=36) compared to pathologically normal breast tissues (Breast, n=61). *$P < 0.05$. $^{\dagger}P = 0.0569$.

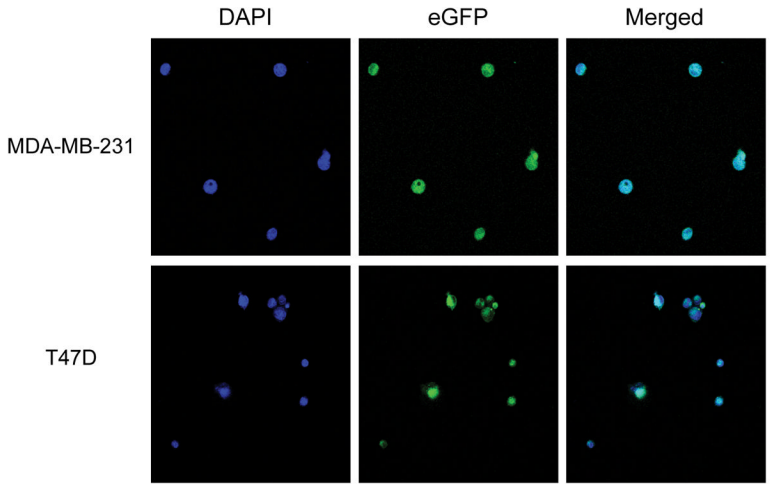

Figure 5. Ectopically-expressed eGFP-MIER3 localizes to nuclei in breast cancer cell lines

Plasmid expression vectors containing eGFP_MIER3 ORFs were transiently transfected into MDA-MB-231 or T47D breast cancer cell lines. At 24 h cells were fixed in formalin and stained with DAPI to visualize nuclei. Confocal microscopy was used to visualize DAPI staining and green fluorescence. Green fluorescence from eGFP-MIER3 and DAPI nuclear staining co-localized in both cell lines (Merged).

Table 1

Mammary carcinoma multiplicity phenotypes (mean mammary carcinomas *per* rat ± SD) by genotype for WF.COP *Chr 2* congenic lines used to map *Mcs1b* to 1.8 Mb

WF.COP *Chr2* region	Line	COP/COP (COP/WF)	n	WF/WF	n	P value*
D2Uwm17/g2UL2-30	T†	3.5±2.2	21	8.3±3.3	19	0.0010
D2Uwm17/D2Ulb4	F3	9.6±4.1	32	8.8±3.5	32	0.8433
D2Mgh2/g2UL1-5	N3	3.4±2.0	25	7.8±3.1	25	0.0001
	N3	(5.5±3.6)	15			0.0413
D2Ulb4/ENSRNOSNP2740854	W2	6.0±1.8	18	5.9±3.2	9	0.8498
D2Rat116/ENSRNOSNP2740854	U2	5.7±3.9	6	6.3±3.3	12	0.8866
g2UL2-27/D2Rat201	I4	9.3±3.0	19	7.9±3.7	13	0.2470

Abbreviations: WF, Wistar Furth; COP, Copenhagen; *Chr*, chromosome

*P values from Mann Whitney tests

†Line T phenotype published previously by Haag *et al. Cancer Research*, 63:5808–5812, 2003

Table 2

Mammary gland graft-site and carcinoma outcome analyses.

Raw data from mammary gland grafting assays				
	***Mcs1b* Donor : Recipient Genotype**			
	S : S	**S : R**	**R : S**	**R : R**
Total recipients (n)	28	25	23	22
MG-graft positive (n)	27	23	18	22
MG-graft AND tumor positive (n)	15	13	4	5

Logistic regression of graft site mammary gland outcome			
Effect	**Coefficient**	**P value**	**Odds Ratio (95% CI)**
Donor	0.99	0.1869	2.69 (0.62 – 11.68)
Recipient	−1.31	0.1160	0.27 (0.05 – 1.38)
Intercept	2.70	0.0004	

Logistic regression of mammary gland graft site tumor outcome			
Effect	**Coefficient**	**P value**	**Odds Ratio (95% CI)**
Donor	1.48	0.0019	4.40 (1.73 – 11.18)
Recipient	−0.04	0.9381	0.96 (0.39 – 2.36)
Intercept	−1.22	0.0045	

Abbreviations: S, susceptible genotype; R, *Mcs1b* resistant genotype; n, number of females; MG, mammary gland; CI, confidence interval

Table 3

Analysis and Statistics of *Mcs1b* Potential Candidate Gene Mammary Gland Transcript Levels in *Mcs1b* Resistant and Susceptible Genotypes at Twelve Weeks of Age

	Two-Way ANOVA F-Test P-Values				Log_2 Target/Rplp2 Mean ± SD (n)		
Target	***Mcs1b* Genotype**	**Exposure**	**G X E**	**Exposure**	**Susceptible**	***Mcs1b* Resistant**	**P value***
Gpbp1	0.0101	0.2090	0.6422	Control	0.586 ± 0.600 (34)	0.044 ± 0.734 (29)	0.0020
				DMBA	0.281 ± 1.309 (45)	−0.097 ± 1.246 (42)	0.1716
Mier3	0.0023	0.7911	0.6682	Control	0.115 ± 0.594 (34)	−0.522 ± 1.278 (34)	0.0104
				DMBA	0.154 ± 1.557 (45)	−0.688 ± 1.943 (48)	0.0240
Map3k1	0.0002	0.0003	0.0588	Control	−0.092 ± 0.818 (34)	−0.725 ± 0.767 (32)	0.0019
				DMBA	0.105 ± 0.564 (47)	−0.104 ± 0.651 (45)	0.1036
Ankrd55	0.4694	0.2025	0.9019	Control	−0.691 ± 0.678 (24)	−0.826 ± 1.108 (22)	0.6180
				DMBA	−0.377 ± 1.296 (17)	−0.567 ± 1.006 (22)	0.6090
Il6st	0.0199	0.1744	0.8435	Control	−0.066 ± 0.755 (36)	−0.418 ± 1.054 (33)	0.1137
				DMBA	0.189 ± 1.006 (44)	−0.227 ± 1.181 (48)	0.0734
Il31ra	0.2869	0.8674	0.9928	Control	−0.331 ± 1.072 (24)	−0.559 ± 0.761 (23)	0.4072
				DMBA	−0.368 ± 0.942 (20)	−0.592 ± 1.159 (23)	0.4949
Ddx4	0.0555	0.5442	0.4045	Control	−0.107 ± 0.983 (36)	−0.359 ± 0.911 (33)	0.2748
				DMBA	−0.055 ± 1.122 (18)	−0.690 ± 1.575 (17)	0.1769
Slc38a9	0.1008	0.3929	0.9730	Control	−0.285 ± 0.600 (24)	−0.575 ± 0.681 (23)	0.1284
				DMBA	−0.144 ± 0.970 (20)	−0.422 ± 0.954 (23)	0.3499
Ppap2a	0.3918	0.8314	0.5788	Control	−0.385 ± 0.632 (24)	−0.447 ± 0.765 (23)	0.7629
				DMBA	−0.315 ± 1.357 (20)	−0.605 ± 1.029 (23)	0.4315

Abbreviations: n, number of females; G, genotype; E, exposure; DMBA, 7,12-dimethylbenz[a]anthracene

*Fisher's PLSD test P values from comparing susceptible and *Mcs1b* resistant genotypes by exposure