Genetically determined inflammatory-response related cytokine and chemokine transcript profiles between mammary carcinoma resistant and susceptible rat strains

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Abstract

Multiple human breast and rat <u>mammary carcinoma susceptibility</u> (*Mcs*) alleles have been identified. Wistar Kyoto (WKY) rats are resistant to developing mammary carcinomas, while Wistar Furth (WF) females are susceptible. Gene transcripts at *Mcs5a1, Mcs5a2,* and *Mcs5c* are differentially expressed between resistant WKY and susceptible WF alleles in immune-system tissues. We hypothesized that immune-related gene transcript profiles are genetically determined in mammary carcinoma resistant and susceptible mammary glands. Low-density QPCR arrays were used to compare inflammation related genes between mammary carcinoma resistant WKY and susceptible WF females. Mammary gland gene transcript levels predicted to be different based on arrays were tested in independent samples. In total, twenty females *per* strain were exposed to 7,12-dimethylbenz(a)anthracene (DMBA) to induce mammary carcinogenesis. Twelve age-matched controls *per* strain without DMBA were included to determine main effects of DMBA-exposure. Significant (ANOVA *P* 0.01) effects of strain on mammary gland transcript level were observed for Cx3cl1, Il11ra, Il4, C3, Ccl20, Ccl11, Itgb2, Cxcl12, and *Cxcr7*. Significant effects of DMBA-exposure were observed for *Cx3cl1, Il11ra, Cxcr4, Il4ra,* and *Il4*. Strain and DMBA-exposure interaction effects were significant for *Cx3cl1*. Transcript levels of *Cxcr7* relative to *Cxcr4* were modified differently by DMBA in mammary carcinoma resistant and susceptible strains. In conclusion, several genetically-determined differences in cytokine, chemokine, and receptor gene transcript levels were identified between mammary carcinoma susceptible and resistant mammary glands, which may be indicative of cell populations and activities that suppress mammary carcinogenesis in resistant genotypes.

Keywords

breast cancer susceptibility; rat mammary carcinogenesis; inflammation; *Ccl20*; *Cxcr7*

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Human breast and rat mammary cancer susceptibility are complex phenotypes. A majority of breast cancer risk associated low penetrance polymorphisms are located in non-coding DNA [1], suggesting genetic variation in gene-regulatory mechanisms is important for determining susceptibility. Mammary carcinoma resistant Wistar Kyoto (WKY) and susceptible Wistar Furth (WF) rat strains have been used to identify rat mammary carcinoma susceptibility (*Mcs*) loci [2]. Some rat *Mcs* loci are known to be concordant orthologs of human breast cancer risk associated or potentially associated alleles [3, 4]. *F-box protein 10* (*Fbxo10*), *FERM and PDZ domain containing 1* (*Frmpd1*), and *tenascin c* (*Tnc*), which are respectively located at rat *Mcs5a1, Mcs5a2*, and *Mcs5c*, are differentially expressed between susceptible and resistant rat alleles in immune cells and tissues [3, 5, 6].

Biological differences between breast cancer susceptibility genotypes that exist *prior* to the formation of palpable tumors are important to identify. Additionally, mechanisms identified in cancer resistant rats may be good targets for cancer prevention in humans. Inflammatory responses play decisive roles at different stages of tumor development, including initiation, promotion, malignant conversion, invasion, and metastasis [7]. Immune-mediated inflammatory cells, cytokines, and chemokines are associated with breast cancer risk, prognosis, and metastatsis [8]. We hypothesized that additional immune system genes may be differentially expressed between mammary cancer resistant WKY and susceptible WF rats. We report and compare genetically determined mammary gland transcript-level differences in inflammatory cytokines, chemokines, and receptors between these genotypes.

We used low-density QPCR arrays that contained eighty-four rat inflammatory cytokine and chemokine genes followed by validation in independent samples. One QPCR array *per* rat sample (N=16 arrays) was used to compare transcript levels between cancer susceptible (n = 8 WF) and resistant (n = 8 WKY) females that were twelve weeks of age and had received a single oral dose of DMBA to induce mammary carcinogenesis. This age was chosen because it is after acute DMBA toxicity and before frank carcinomas are detectable by palpation in cancer susceptible WF mammary glands. Inflammatory cytokines, chemokines, and receptors that were differentially expressed ($P < 0.05$) between these strains in this experiment were: *Cx3cl1, Il4, C3, Cxcl12/Sdf1*, and *Cxcl4/Pf4*. Those approaching significant differential expression ($P > 0.05$ and < 0.25) between resistant and susceptible mammary glands were: *Ccl20, Ccl11/eotaxin, Itgb2/Cd18, Il11, Il8rb*, and *Cxcl1*. Complete gene names are listed in Table S1.

Further testing was completed to determine which predictions between strains were valid, and to compare gene transcript levels between mammary carcinogen induced (DMBA) and age-matched unexposed mammary glands. Receptors for chemokine Cxcl12 (Cxcr4, Cxcr7) and cytokines Il11 (Il11ra) and Il4 (Il4ra) were not present on QPCR arrays used; therefore, transcript levels of these receptors were tested in this experiment. Receptors of other cytokines and chemokines that data suggested to be different were present on QPCR arrays, and were not significantly different between DMBA-exposed susceptible and resistant rat mammary glands.

We used mammary gland total RNA collected from a second group of twelve-week old female rats that were administered DMBA independently of females used in our QPCR array experiment. Mammary carcinoma susceptible (n = 24 WF) and resistant (n = 24 WKY) females were randomly assigned to two exposure groups, with DMBA (n = 24 females) and without (n = 24 females). Samples from DMBA-exposed females used on low-density QPCR arrays were also tested with primers designed for this experiment and statistically analyzed with samples from this second group of females. Quantitative PCR results shown in Figure 1A were analyzed by two-way ANOVA for independent effects of strain and DMBA-exposure, and an interaction effect (Table 1). Cytokines, chemokines, and

receptors with a statistically significant effect of strain were *Cx3cl1* ($P < 0.0001$), *Il11ra* ($P = 0.0023$), *Il4* ($P = 0.0025$), *C3* ($P < 0.0001$), *Ccl20* ($P < 0.0001$), *Ccl11/eotaxin* ($P < 0.0001$), *Itgb2/Cd18* ($P = 0.0018$), *Cxcl12/Sdf1* ($P = 0.0004$), and *Cxcr7* ($P = 0.0112$). With exception to *Ccl20*, transcripts that were significantly different were 1.7 to 4-fold higher in cancer resistant compared to susceptible mammary glands. Chemokine *Ccl20* transcript levels were 11.5-fold higher in mammary glands from susceptible compared to resistant females.

Cytokines, chemokines, and receptors with a significant effect of DMBA-exposure (with or without) on mammary gland transcript level were *Cx3cl1* ($P < 0.0001$), *Il11ra* ($P = 0.0024$), *Il4* ($P = 0.0036$), *Cxcr4* ($P < 0.0001$), *Il4ra* ($P = 0.0026$). Transcript level of *Il11ra* was 1.8 fold higher with DMBA compared to without. All other transcripts with a significant exposure effect were decreased 24-64% with DMBA compared to without.

A strain by DMBA-exposure interaction was statistically significant for *Cx3cl1* ($P < 0.0001$; Table 1). Mammary gland *Cx3cl1* levels between resistant and susceptible females shown in Figure 1B were 3.3-fold higher in mammary carcinoma resistant females without DMBA ($P = 0.0034$), but were not different between strains with DMBA ($P = 0.7483$). The source of a potential *Cxcr7* strain by exposure interaction ($P = 0.0435$) was a *Cxcr7* transcript level increase (Figure 1B) within resistant mammary glands exposed to DMBA ($P = 0.0328$, one-tailed t-test), and an observed decrease in susceptible mammary glands ($P = 0.1238$, one-tailed t-test).

CXCR4 and CXCR7 are both G-protein coupled receptors of CXCL12. It seemed from our data that potential complexity existed between mammary cancer susceptible and resistant genotypes for these two receptors. We completed *a posteriori* tests to determine if interactions existed between these two genes. Mammary gland *Cxcr4* and *Cxcr7* transcript level was used as the dependent variable in two-way ANOVA. Gene ($P = 0.0137$), DMBA-exposure ($P = 0.0001$), and the interaction ($P = 0.0036$) were statistically significant when mammary cancer susceptible WF females were analyzed. The gene by exposure interaction only was significant ($P = 0.0013$) when mammary cancer resistant *Cxcr4* and *Cxcr7* transcript levels were used as the dependent variable. As displayed in Figure 1C, levels of *Cxcr7* relative to *Cxcr4* were lower in both susceptible ($P = 0.0367$) and resistant ($P = 0.0606$) mammary glands that had not been exposed to DMBA; however, four weeks after DMBA exposure *Cxcr7* levels were higher than *Cxcr4* levels in resistant mammary glands ($P = 0.0059$). This was due to a drop in *Cxcr4* and an increase in *Cxcr7* in resistant mammary glands following carcinogen exposure. As in the resistant strain, levels of *Cxcr4* dropped in susceptible mammary glands following DMBA exposure, but *Cxcr7* levels did not increase relative to *Cxcr4* levels ($P = 0.4673$).

In summary, transcript levels of *Cx3cl1, Il11ra, Il4, C3, Ccl11, Itgb2, Cxcl12*, and, *Cxcr7* were higher, and *Ccl20* levels were lower in mammary carcinoma resistant compared to susceptible mammary glands. These genetically determined differences in cytokine, chemokine, and receptor profiles between susceptible and resistant genotypes are anticipated to lead to differences in mammary gland stromal constitutes and activities. Further, these differences may be important for a cancer resistant mammary gland to launch an immediate response to carcinogenesis. Cytokine and chemokine transcript profiles between mammary carcinoma resistant and susceptible mammary glands suggest that lymphocytes, eosinophils, neutrophils, denditric, and NK cells may be contributing to susceptibility differences between these strains. In support of our results, Smits *et al.* reported that genetically determined mammary gland $_{\gamma\delta}$T cell population differences exist between mammary carcinoma resistant WF.WKY-*Mcs5a* congenic and susceptible females [9]. Chemokine CX3CL1/fractalkine is produced by a variety of cells, recruits Th effector cells, and

promotes the adhesion of monocytes to endothelial cells [10]. Chemokine CCL20 is expressed in cytokeratin positive cells and potentially recruits dendritic cell precursors [11]. Depending on subtype, dendritic cells may inhibit or promote tumorigenesis [12].

Chemokine CCL11/eotaxin attracts eosinophils, which are important for proper mammary gland development as *Ccl11*^{-/-} mice have deficiencies in mammary gland branching and terminal end bud formation [13]. Blomqvist and colleagues reported that eosinophils were not detected in any of over 200 hematoxylin-eosin stained invasive breast cancer tissues [14]. Another link to a potential for eosinophil mediated cancer resistance is Il4, a pleiotropic, anti-inflammatory, and anti-tumorigenic cytokine produced by Th2 CD4+ T cells, eosinophils, basophils, mast cells, and NKT cells. Wu *et al.* discovered that eosinophils are the major Il4 producing cells in mouse white adipose tissue [15]. Another potential source of increased *Il4* transcript levels in WKY cancer resistant mammary glands may be Il4 producing $_{\gamma\delta}$T cells, which are at higher levels in WF.WKY-*Mcs5a* compared to cancer susceptible mammary glands [9]. Importantly, IL4 may also promote tumorigenesis depending on the source, dose, and window of expression of IL4 [16].

To date, none of the genes identified in this study have been determined to be rat mammary carcinoma susceptibility genes. The WKY rat strain used in this study is completely resistant to developing mammary carcinomas; however, it does harbor increased susceptibility alleles that include *Mcs5b* and *Mcs8* [2, 17]. Therefore, additional work will be required to determine which transcripts represent a favorable condition when at a higher level and which might be unfavorable. Studies will also be required to determine if these genes segregate with mammary carcinoma susceptibility. Interestingly, none of the genetically determined transcript profiles identified in this study are located within known WKY resistant-rat *Mcs* QTLs (Figure S1). This suggests that if these gene transcripts do contribute to mammary cancer susceptibility, either they are controlled in *trans* by known *Mcs* QTLs or unknown WKY *Mcs* QTLs exist. It is noteworthy that even though *Cxcl1* and *Cxcl4* did not meet our criterion for statistical significance, these transcripts are located within *Mcs8*. Further investigation of *Cxcl1* and *Cxcl4* is warranted as subtle transcript level differences have been associated with *Mcs5a1, Mcs5a2*, and *Mcs5c* alleles [3, 5]. *Interleukin 11ra* is located within rat *Estrogen induced mammary cancer-8*, a QTL identified using the ACI rat strain, which is susceptible to 17β-estradiol induced mammary cancer [18].

Human orthologous loci containing these genes are good candidates to test in genome-targeted breast cancer risk association studies. Epidemiological evidence indicates that *C3*, an estrogen responsive gene, is associated with increased breast cancer risk in nulliparous compared to parous women [19]. Genetic variation at *ITGβ2/CD18* and *CXCL12* loci has shown association or potential-association with breast cancer susceptibility [20, 21]. Breast cancer cells that express ITGβ2 are recognized and killed my natural killer cells [22]. Chemokine gene *CXCL12* is estrogen regulated and the gene product attracts naïve T cells and monocytes [23].

In our study transcript levels of *Cx3cl1, Il11ra, Il4, Cxcr4*, and *Il4ra* were significantly different between DMBA-exposed and unexposed mammary glands. These differences are suggestive of responses to early stages of mammary carcinogenesis, as mammary gland transcript levels were evaluated before frank carcinomas are detectable in mammary carcinoma susceptible rats.

In conclusion, we identified multiple inflammation-related cytokines, chemokines, and receptors with different transcript levels between mammary carcinoma susceptible and resistant mammary glands. We also uncovered an interaction between *Cxcr4* and *Cxcr7* transcript levels in a carcinoma resistant mammary gland. A majority of differentially

expressed gene transcripts identified (all except *Ccl20*) were higher in resistant compared to susceptible mammary glands, suggesting that mammary cancer resistance is an active process. Although some gene transcript levels decreased in response to DMBA they remained higher in resistant compared to susceptible genotypes. Our results support a hypothesis that genetically determined differences in immune-cell populations or activities exist within mammary glands in response to events related to carcinogenesis.

Materials and Methods

Mammary carcinoma susceptible Wistar Furth (WF/NHsd) and resistant Wistar Kyoto (WKY/NHsd) virgin female rats were purchased from Harlan (Indianapolis, IN). Procedures involving rodents were carried out in accordance to protocols approved by the University of Louisville Animal Care and Use Committee. Rats were housed in an AAALAC approved facility with a 12 hour light/dark cycle and *ad libitum* food/water. To induce mammary carcinogenesis, 7,12-Dimethylbenz(a)anthracene (DMBA) was dissolved in sesame oil at 20 mg/mL and 65 mg DMBA/KG body mass was administered orally by gavage between 50-55 days of age.

Right cranial inguinal mammary glands with lymph nodes removed were excised four weeks following DMBA administration, flash frozen in liquid nitrogen, and stored at -80° C. Total RNA was isolated from mammary gland tissue homogenates using TRI reagent (Molecular Research Center), potential genomic DNA contamination was reduced using TURBO DNase (Life Technologies), and one g RNA was used to generate cDNA using SuperScript III reverse transcriptase (Life Technologies). One rat inflammatory cytokine QPCR array (SABiosciences PN PARN011, Qiagen) *per* sample was used to screen 84 genes involved in mediating an inflammatory response. Validation by SYBR-green (Life Technologies) QPCR was completed using primers designed with Primer-3 [24] and independent groups of WF and WKY rats with DMBA and without. Primers used for QPCR are provided in Table S2. Relative quantification (RQ) values were used to compare transcript levels. RQ is the expression level of a nucleic acid sequence (target) in an experimental sample relative to a calibrator sample. Rat *Rplp2* was used as an endogenous control to standardize target levels.

Statistical significance level of each low-density array comparison was determined using a Student's *t*-test and SA Biosciences software (Qiagen). Statistical analysis of data to validate predictions based on low-density arrays was performed by two-way ANOVA. Here RQ of gene transcript level was the dependent variable. Strain (WKY, WF) and DMBA-exposure (with, without) were independent variables. Two-way ANOVA was also used to test for an interaction between *Cxcr4* and *Cxcr7* gene transcript levels. RQ of gene transcript level was the dependent variable. Gene (*Cxcr4, Cxcr7*) and DMBA-exposure (with, without) were independent variables. To reduce potential for false-positives a p value   0.01 was considered statistically significant. Student's *t*-tests were used for *a posteriori* comparisons of transcript levels between two groups. Unless denoted otherwise, significance level probabilities are two-tailed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

DMBA	7,12-dimethylbenz(a)anthracene
Mcs	mammary carcinoma susceptibility
RQ	relative quantification
WKY	Wistar Kyoto
WF	Wistar Furth
QPCR	quantitative polymerase chain reaction

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Highlights

- We compared inflammatory genes in a rat genetic model of human breast cancer.

- Most differentially expressed transcripts were higher in resistant mammary glands.

- Transcripts different in carcinogen exposed/un-exposed females were identified.

- Mammary *Cxcr7* was higher relative to *Cxcr4* in carcinogen exposed resistant females.
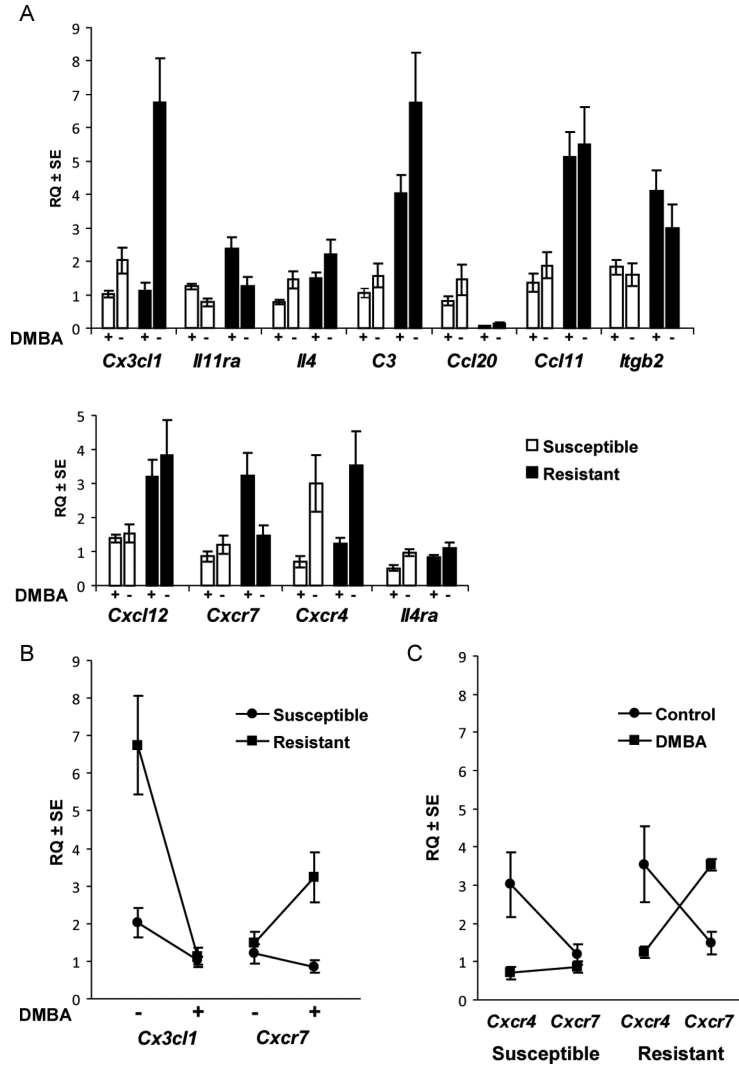
Figure 1. Inflammatory cytokine, chemokine, and receptor transcript levels in mammary carcinoma susceptible WF and resistant WKY mammary glands with DMBA and without
Graphed are means ± SEs of relative quantification (RQ) of genes indicated on x-axes. *Rplp2* levels were used to standardize targets. Twenty females *per* strain were given DMBA, and twelve aged matched controls *per* strain were included. **A**, inflammatory cytokine, chemokine, and receptor mammary gland transcript levels in twelve week old mammary carcinoma susceptible WF (unfilled bars) and resistant WKY (filled bars) females with DMBA [+] or without [-]. **B**, strain by DMBA-exposure interactions were statistically significant for *Cx3cl1* ($P < 0.0001$) and potentially significant for *Cxcr7* ($P = 0.0435$). Compared to mammary cancer susceptible WF mammary glands (circles), *Cx3cl1* expression was significantly higher in mammary glands from resistant WKY rats (squares) not exposed to DMBA ($P = 0.0034$). There was no difference in *Cx3cl1* transcript level between strains in mammary carcinogen induced (DMBA +) females ($P = 0.7483$). *Cxcr7* levels were significantly higher in mammary cancer resistant WKY rats (squares) compared susceptible WF females (circles) with DMBA ($P = 0.0027$), but not without ($P = 0.4966$). **C**, mammary gland *Cxcr4* and *Cxcr7* expression in DMBA-exposed (squares) and un-exposed (circles) females compared between mammary carcinoma resistant WKY and susceptible WF rat strains. *Cxcr4* expression was higher than *Cxcr7* in both strains ($P = 0.0367$ [WF] and 0.0606 [WKy]) when control females not exposed to mammary carcinogen were

evaluated. *Cxcr7* levels were significantly higher than *Cxcr4* levels in the mammary cancer resistant strain (WKY) following DMBA exposure ($P = 0.0059$). *Cxcr7* and *Cxcr4* transcript levels were similar in mammary cancer susceptible WF females following DMBA exposure ($P = 0.4673$).

Table 1

ANOVA Summary and Fold Differences in Mean Cytokine, Chemokine, and Receptor Transcript Levels Between Mammary Carcinoma Resistant WKY and Susceptible WF Rat Strains With DMBA and Age-Matched Controls Without

Gene Transcript	ANOVA P-Value*			Transcript Level Fold-Difference (Resistant/Susceptible)			Transcript Level Fold-Difference (DMBA/Control)
	Strain (S)	Exposure (E)	G × E	DMBA	Control	Combined	
Cx3cl1	<0.0001	<0.0001	<0.0001	1.09	3.33	2.28	0.24
Il11ra	0.0023	0.0024	0.2053	1.90	1.62	1.84	1.82
Il4	0.0025	0.0036	0.9258	1.91	1.52	1.73	0.61
C3	<0.0001	0.0238	0.1204	3.84	4.30	4.06	0.60
Ccl20	<0.0001	0.0557	0.1182	0.08	0.09	0.09	0.57
Ccl11/eotaxin	<0.0001	0.5322	0.9123	3.77	2.92	3.41	0.87
Itgb2/Cd18	0.0018	0.2305	0.4324	2.24	1.86	2.11	1.29
Cxcl12/sdf1	0.0004	0.4826	0.6529	2.29	2.49	2.37	0.85
Cxcr7	0.0112	0.1688	0.0435	3.76	1.23	2.62	1.59
Cxcr4	0.2897	<0.0001	0.9889	1.78	1.18	1.46	0.29
Il4ra	0.0573	0.0026	0.4653	1.61	1.15	1.42	0.64
Cxcl4/Pf4	0.0552	0.3317	0.2880	1.74	1.21	1.55	1.21
Il1	0.0617	0.7137	0.7956	1.29	1.22	1.26	1.04
Il8rb	0.7704	0.0137	0.5604	1.04	0.91	0.99	0.73
Cxcl1	0.1177	0.0354	0.1226	0.99	0.41	0.62	0.43

DMBA: 7,12-Dimethylbenz(a)anthracene given at 50-55 days of age; QPCR was used to compare transcript levels between rat mammary glands from 12 week old mammary carcinoma resistant (WKY) and susceptible (WF) virgin females with DMBA and controls without DMBA

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P-Values from two-way ANOVA of gene transcript level by DMBA exposure (with or without), mammary carcinoma susceptibility genotype (resistant or susceptible), and interaction (S × E).