Cytokine 59 (2012) 223–227

Contents lists available at SciVerse ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/issn/10434666

Short Communication

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

Bharat Devapatla [a,b], Jennifer Sanders [a], David J. Samuelson [a,b,*]

[a] Center for Genetics & Molecular Medicine, Department of Biochemistry & Molecular Biology, University of Louisville School of Medicine, Louisville, KY, USA
[b] James Graham Brown Cancer Center, University of Louisville School of Medicine, Louisville, KY, USA

ARTICLE INFO

*Article history:*
Received 16 February 2012
Received in revised form 13 April 2012
Accepted 23 April 2012
Available online 18 May 2012

*Keywords:*
Breast cancer susceptibility
Rat mammary carcinogenesis
Inflammation
*Ccl20*
*Cxcr7*

ABSTRACT

Multiple human breast and rat mammary carcinoma susceptibility (*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, 20 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 $P \leqslant 0.01$) 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.



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 metastasis [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

*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.

* Corresponding author. Address: Center for Genetics & Molecular Medicine, Department of Biochemistry & Molecular Biology, University of Louisville, Health Sciences Center, A Room 708, 319 Abraham Flexner Way, Louisville, KY 40292, USA. Tel.: +1 502 852 7797; fax: +1 502 852 6222.
*E-mail address:* david.samuelson@louisville.edu (D.J. Samuelson).

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http://dx.doi.org/10.1016/j.cyto.2012.04.037

determined mammary gland transcript-level differences in inflammatory cytokines, chemokines, and receptors between these genotypes.

We used low-density QPCR arrays that contained 84 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 12 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 12-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 Fig. 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 1.7–4-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 Fig. 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 (Fig. 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 Fig. 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, 4 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, dendritic, 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