CXM: A New Tool for Mapping Breast Cancer Risk in the Tumor Microenvironment

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Abstract

The majority of causative variants in familial breast cancer remain unknown. Of the known risk variants, most are tumor cell autonomous, and little attention has been paid to germline variants that may affect the tumor microenvironment. In this study, we developed a system called the Consomic Xenograft Model (CXM) to map germline variants that affect only the tumor microenvironment. In CXM, human breast cancer cells are orthotopically implanted into immunodeficient consomic strains and tumor metrics are quantified (e.g., growth, vasculogenesis, and metastasis). Because the strain backgrounds vary, whereas the malignant tumor cells do not, any observed changes in tumor progression are due to genetic differences in the nonmalignant microenvironment. Using CXM, we defined genetic variants on rat chromosome 3 that reduced relative tumor growth and hematogenous metastasis in the SS. BNL225 consomic model compared with the SSIL2R parental strain. Paradoxically, these effects occurred despite an increase in the density of tumor-associated blood vessels. In contrast, lymphatic vascularature and lymphogenous metastasis were unaffected by the SSBN3IL2R background. Through comparative mapping and whole-genome sequence analysis, we narrowed candidate variants on rat chromosome 3 to six genes with a priority for future analysis. Collectively, our results establish the utility of CXM to localize genetic variants affecting the tumor microenvironment that underlie differences in breast cancer risk. Cancer Res; 74(22); 6419–29. © 2014 AACR.

Introduction

Breast cancer is the most prevalent female malignancy (http://apps.nccd.cdc.gov/uscs/) and is highlyheritable (1–4), yet approximately 72% of breast cancer risk remains undefined (5). Heritable factors underlie most aspects of breast cancer risk [e.g., incidence (5), age of onset (6), metastatic progression (7), and disease-free survival (8)]. In addition to variants that affect tumor cells directly (i.e., tumorigenicity), heritability is implicated in multiple components of the tumor microenvironment [e.g., tissue remodeling (9), angiogenesis (10, 11), and immunity (12)], which also affect tumorigenesis and progression. However, the genetic variants underlying differences in the tumor microenvironment have rarely been the focus of genetic mapping studies and as such remain poorly defined.

We have developed a new model of breast cancer (termed the Consomic Xenograft Model; CXM) that focuses on genetic mapping of strain-specific variants that affect tumor progression through the tumor microenvironment. A consomic rat is one in which an entire chromosome is introgressed into the isogenic background of another inbred strain by selective breeding (13). Thus, observed phenotypes can be linked to single chromosomes and then further elucidated by comparative sequence analysis and/or selective backcrossing to yield smaller congenics (13). In CXM, the consomic and parental strains are converted to SCID (severe combined immunodeficiency), so that orthotopically xenografted human breast cancer cells can be tested in vivo. Because the human breast cancer cells are not varied between strains, any differences in breast cancer progression (e.g., primary site growth,
vasculogenesis, and distal metastasis) are due solely to genetic differences in the tumor microenvironment, not the malignant cancer cells. CXM utilizes transgenically tagged human cancer cells with defined properties (e.g., triple-negative, prometastatic, etc.). Thus, it enables testing of clinically relevant cancer models in strain backgrounds with varying genetic predispositions to breast cancer. Finally, we have developed CXM in the rat, but postulate that this technique could also be applied to the mouse and other model species.

For purposes of illustration, the basic concept of CXM is outlined in Fig. 1. In brief, CXM utilizes transcription activator-like effector nucleases (TALEN; ref. 14) to generate SCID on any strain background by mutating the \( IL2R_g \) gene (15). We first generated and characterized an \( IL2R_g \)-mutant parental SS strain (SS\(^{IL2R_g}\)), which was then selectively bred onto the SS.

BN3 consomic background to generate the SS.BN3\(^{IL2R_g}\) consomic. The parental SS (SS/JrHsdMcwi) rat strain is susceptible to mammary tumors, whereas the BN (BN/NHsdMcwi) rat strain is highly resistant to mammary tumors (16). Our rationale for choosing SS (parental) and SS.BN3 consomic (i.e., BN chromosome 3 introgressed onto the isogenic SS background) was based on prior data demonstrating a 64% reduction in breast cancer incidence in the SS.BN3 consomic compared with the SS (16) and other evidence that multiple overlapping protective loci exist on rat chromosome 3 (16–19). We hypothesized that the protective effects of BN chromosome 3 could be in part due to changes in the tumor microenvironment. To test this hypothesis, luciferase-tagged human MDA-MB-231 breast cancer cells (231Luc\(^+\)) were orthotopically implanted and tracked for tumor growth,

Figure 1. Overview of the CXM. A, reduced susceptibility to mammary tumors (DMBA-induced) in SS.BN3 consomic rats (n = 14) compared with SS (n = 40), as reported previously by Adamovic and colleagues (16). ***, \( P < 0.001 \), as determined by the Fisher exact test using Bootstrap. B, schematic representation of the SS and SS.BN3 genomes that were modified by TALEN-mediated editing of the \( IL2R_g \) gene. The numbered bars represent chromosomes that are derived from SS (white) or BN (black). Note that the only genetic differences between SS\(^{IL2R_g}\) and SS.BN3\(^{IL2R_g}\) are the inheritance of chromosome 3 from the SS or BN rats. C, the transgenically labeled human 231Luc\(^+\) breast cancer cells were orthotopically implanted in the MFP (indicated by dashed lines) of SS\(^{IL2R_g}\) and SS.BN3\(^{IL2R_g}\) rats. Tumor progression (e.g., growth, vasculogenesis, and metastasis) were tracked over the duration of the experiment. D, because the 231Luc\(^+\) tumor cells are the same between strains (gray), any differences in tumor progression can be attributed to differences in the SS\(^{IL2R_g}\) (green) and SS.BN3\(^{IL2R_g}\) (blue) microenvironments.
vasculogenesis, and distal metastasis. Using CXM, we demonstrate that genetic variants on rat chromosome 3 affect the tumor microenvironment by causing blood vessel-specific defects that attenuate tumor growth and decrease hematogenous metastasis, whereas lymphatic vasculature and lymphogenous metastasis were unaffected. Finally, we used comparative analysis and whole-genome sequencing (WGS) of five rat strains (BN/JrHsdMcwi, Cop/Crl, F344/N, ACI/Eur, and SS/JrHsdMcwi) to prioritize cosegregating candidate genes on rat chromosome 3 for future analysis by gene editing or congenic mapping.

Materials and Methods

Generation of SS.BN3IL2Rf and SSfIL2Rf rat strains

All procedures and protocols were approved by the Medical College of Wisconsin (MCW, Milwaukee, WI) IACUC committee. The IL2Rf gene was targeted in the SS/JrHsdMcwi rat by TALEN injection into single-cell rat embryos, as described previously (20). Once established, a homozygous female rat from the SSfIL2Rf line was intercrossed with a homozygous SS.BN3 male to yield heterozygous SS.BN3IL2Rf offspring (F1), followed by brother-sister mating to yield homozygous SS.BN3IL2Rf offspring from the F2 generation.

Tumor implantation

Luciferase-tagged MDA-MB-231 (231Luc−) cells were orthotopically implanted and measured as described previously (21), with slight modifications. Briefly, 231Luc− cells (6 × 10⁶) in 50% Matrigel were orthotopically implanted into the mammary fat pads (MFP) of 4- to 6-week-old female SSfIL2Rf (n = 11) and SS.BN3IL2Rf (n = 19) rats. Tumor volumes were measured weekly by calipers, as described previously (21).

Detection of lung metastasis by ex vivo luminescent imaging

Rats were intraperitoneally injected with 200 μL of p-luciferin (Promega) diluted in sterile saline (40 mg/mL). The substrate was allowed to circulate for 5 minutes before rats were euthanized and lungs were removed for bioluminescence imaging using the Xenogen Ivis Lumina (Caliper Life Sciences).

Detection of metastatic burden by luciferase activity

Ipsilateral axillary lymph nodes and lungs were excised, washed in PBS, and homogenized in 0.2 and 2 mL of cold cell culture lysis reagent buffer (Promega), respectively. Cell debris was removed by centrifugation. Protein concentrations of cleared lysates were determined by Bradford assay (Bio-Rad). Fifty microliters of Luciferase Assay Reagent (Promega) was mixed with 10 μL of lysate, and a 10-second average of luminescence was detected using a Varioskan Flash luminometer (ThermoFisher). Cell culture lysis reagent buffer without tissue homogenates and lymph nodes or lungs of non-tumor-bearing rats was used to calculate background and subtracted from the results. The net results are expressed as relative light units normalized per milligram of total protein.

Histologic detection of lung metastasis

Metastatic lesions in the lungs of tumor-bearing SSfIL2Rf (n = 5) and SS.BN3fIL2Rf (n = 5) rats were also assessed by hematoxylin and eosin (H&E) staining, as described previously (22). Formalin-fixed lung sections were H&E stained using a Tissue-Tek Prisma Automated Stainer (Sakura) according to the manufacturer’s protocol. Following H&E staining, three serial sections per lung were examined by two blinded observers.

Matrigel plug angiogenesis assay

Female SS (n = 9) and SS.BN3 (n = 19) rats at 6–8 weeks of age were implanted in the MFP with 500 μL of 100% Matrigel (cat. CB-40234; BD Biosciences) supplemented with 500 ng/mL of recombinant rat VEGFA164 purchased (R&D Systems). At 5 days postimplantation, Matrigel plugs were excised, snap-frozen in optimum cutting temperature medium (Tissue Tek), and stained with anti-CD31 as described in the Supplementary Materials and Methods.

Blood and lymphatic vessel densities

231Luc− tumors and Matrigel plugs were excised, frozen-sectioned, and immunostained with antibodies against the blood vessel marker, CD31, or the lymphatic vessel marker, LYVE-1, as described previously (23). To quantify tumor blood vessel density (BVD) and lymphatic vessel density (LVD), 3 to 4 representative images of CD31+ structures (BVD) and LYVE-1+ structures (LVD) per tissue were acquired at ×100 magnification using a Nikon E400 microscope equipped with a Spot Insight camera (Nikon Instruments). BVD and LVD, average number of vessels per area of the field ± SEM (n = 5–7 rats/group).

Quantification of BVD in DMBA-induced mammary tumors

Acquisition of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors from the SS.BN3 consomic (n = 9) and SS (n = 9) rats were previously described by Adamovic and colleagues (16). These tumors were previously formalin-fixed and paraffin-embedded, precluding us from using the traditional anti-CD31 antibody that does not recognize formalin-fixed antigens. To circumvent this issue, DMBA-induced mammary tumors were stained with anti-SH2B3 antibodies (Sigma Aldrich; cat. HPA005483), which we have demonstrated to specifically recognize CD31 blood vessels, but not LYVE1+ lymphatic vessels (Supplementary Fig. S2). The area of SH2B3+ blood vessels across the entire tumor cross section was acquired at ×100 magnification on a Nikon Eclipse 80i microscope (Nikon Instruments) equipped with an automated stage and a QImaging Micropublisher camera. Images were analyzed using custom Matlab code (MathWorks) that stitched together tissue photographs (~100–750 per sample) and segmented the tissue into to differentiate positively stained vessels (Fig. 4C). The histology segmentation process included a white background correction and contrast optimization. Static thresholds were applied to each photo for vessel segmentation. Relative vascular density was then calculated for each sample and normalized to the entire cross-sectional tumor area.
Peripheral blood analysis
Whole blood from anesthetized rats was analyzed by automated complete blood counts (Marshfield Laboratories) and flow cytometry was performed, as described previously (24, 25).

Genomic sequencing and analysis
Genomic sequence was accessed from the Rat Genome Database (http://rgd.mcw.edu/) and has been described in detail elsewhere (26–28).

Statistical analysis
Statistical analyses were performed using Sigma Plot 11.0 software. Data are presented as mean ± SEM. All data were analyzed by an unpaired Student t test.
Detailed Materials and Methods are provided in the Supplementary Data.

Results

Consomic xenograft model
Traditional mapping studies localize cancer risk variants (29), but do not differentiate between variants that affect malignant cells directly versus those that affect the tumor microenvironment. Here, our goal was to develop a new genetic model of breast cancer risk (i.e., CXM), whereby genetic variants that specifically affect breast cancer progression through the tumor microenvironment are mapped.

Following strain generation (SSIL2R and SS.BN3IL2R), we first examined gross organ morphologies and peripheral blood phenotypes to confirm SCID status and noted strain-dependent differences. No differences in viability, body weight, organ weight and morphology, and some peripheral blood phenotypes (T-cells, B-cells, and NK cells) were observed between SSIL2R (n = 7) and SS.BN3IL2R (n = 11) rats (Supplementary Fig. S1). Differences were observed in circulating monocytes of SS.BN3IL2R (0.4 ± 0.01 per μL blood, P < 0.001) compared with SSIL2R rats (0.17 ± 0.02 per μL blood); in neutrophils of SS.Br3IL2R (1.9 ± 0.3 per μL blood, P < 0.001) compared with SSIL2R rats (4.3 ± 0.4 per μL blood); and in total WBCs of SS.BN3IL2R (2.4 ± 0.4 per μL blood, P < 0.001) compared with SSIL2R rats (5.6 ± 0.5 per μL blood; Supplementary Table S1). Compared with the immunocompetent parental SS and SS.BN3 strains, circulating lymphocytes were reduced 4- to 9-fold (P < 0.001) in SSIL2R and SS.BN3IL2R (Supplementary Table S1), whereas neutrophils were increased by 5- to 9-fold (P < 0.001) in SS.BN3IL2R and SSIL2R compared with the parental strains (Supplementary Table S1). Similar changes in leukocytes have been reported in other immunodeficient models [e.g., SCID rat (15) and SCID mouse (30)]. Taken together, these data demonstrate that SSIL2R and SS.BN3IL2R are both SCID, with limited strain-dependent differences in immunity.

Tumor growth
Previously, we showed that the SS.BN3 consomic rat has significantly reduced tumorigenicity in a DMBA-inducible model of breast cancer (16). We postulated that this could be at least partially due to genetic differences in the tumor microenvironment, rather than inherent differences in the malignant tumor cells only. To test this possibility, 231Luc+ (6 × 10^6 cells) were orthotopically implanted in the MFP of SSIL2R and SS.BN3IL2R rats and tumor growth was measured by caliper measurement at 10, 17, and 24 days postimplantation. Compared with 231Luc+ tumors in SSIL2R rats at 24 days post-implant (5,117 ± 1,038 mm^3, n = 11), tumors grown in SS.BN3IL2R rats (2,616 ± 624 mm^3, n = 19) were roughly 2-fold smaller (P < 0.05; Fig. 2), suggesting that BN-derived antitumor variants or SS-derived protumor variants reside on rat chromosome 3 and function through the tumor microenvironment.

Tumor angiogenesis and hematogenous metastasis
To assess BVD, 231Luc+ tumors that were orthotopically implanted in MFPs of SSIL2R (n = 5 rats) and SS.BN3IL2R (n = 6 rats) rats were excised at 24 days postimplantation and stained with antibodies against the blood vessel marker, CD31 (23, 31) (Fig. 3A). Compared with tumor BVD in SSIL2R rats (101 ± 11 vessels/field), the BVD in SS.BN3IL2R tumors was significantly increased by 27% (130 ± 18 vessels/field; P < 0.05; Fig. 3B). Despite increased BVD in SS.BN3IL2R tumors, we observed fewer blood vessels with open lumens in SS.BN3IL2R tumors (3.2 ± 1.2%; P < 0.05) compared with tumors implanted in SSIL2R rats (6.2 ± 2.0%; Fig. 3C), suggesting that although SS.BN3IL2R had a higher BVD, a greater percentage of these vessels were collapsed and nonfunctional. A similar phenotype (i.e., high density of malformed vessels) was observed by Pandey and Wendell (32) in estrogen-induced pituitary tumors from an overlapping F344.BN-Edpm3BN congenic rat with a 75.7-Mb congenic interval (chr12:36.6-112.3 Mb), suggesting that the vascular defects might be due to BN-derived variants in a common region of rat chromosome 3.

We also observed a small percentage of CD31+ tumor blood vessels being invaded by 231Luc+ tumor cells in both SSIL2R and to a lesser degree in SS.BN3IL2R rats, demonstrating that
Figure 3. Characterization of tumor-associated blood vessels and hematogenous metastasis in 231Luc+ tumors implanted in SS.BN3IL2R and SSIL2R rats at 24 days postimplantation. A, visualization of tumor-associated blood vessels using anti-CD31 staining of 231Luc+ tumors implanted in SS.BN3IL2R and SSIL2R rats. Images were acquired at ×100 magnification. Scale bar, 100 μm. B, mean blood vessel density in 231Luc+ tumors implanted in SS.BN3IL2R and SSIL2R rats was calculated from three images per tumor (n = 5–6 tumors per strain) acquired at ×100 magnification. Data, mean vascular density per ×100 field ± SEM. **, P < 0.05, as determined by the unpaired Student t test. C, percentage of open lumens in 231Luc+ tumors implanted in SS.BN3IL2R and SSIL2R was calculated from three images per tumor (n = 5–6 tumors per strain) acquired at ×100 magnification. Data, the percentage of CD31+ vessels with open lumens per ×100 field ± SEM. **, P < 0.05, as determined by the unpaired Student t test. D, percentage of CD31+ vessels invaded by 231Luc+ cells in tumors implanted in SS.BN3IL2R and SSIL2R was calculated from three images per tumor (n = 5–6 tumors per strain) acquired at ×100 magnification. Data, the percentage of CD31+ vessels with open lumens per ×100 field ± SEM. ***, P < 0.001, as determined by the unpaired Student t test. E, representative luminescent imaging of lungs from tumor-bearing SSIL2R and SS.BN3IL2R rats. F, hematogenous metastatic burden was measured by luminescence activity normalized to total milligrams of protein in lung lysates from SSIIL2R (n = 12) and SS.BN3IL2R (n = 16) rats. Metastatic burden of individual rats is represented by the dots, and the black bars indicate the average metastatic burden per strain. **, P < 0.05, as determined by the unpaired Student t test.

tumor cells were actively undergoing hematogenous metastasis. Quantification of CD31+ blood vessels invaded by 231Luc+ cells revealed a significant decrease of vascular invasion in SS.BN3IL2R tumors (0.8 ± 0.5%; P < 0.001) compared with SSIL2R (2.5 ± 0.5%; Fig. 3D), suggesting that 231Luc+ have reduced metastatic potential in the SS.BN3IL2R rat that is blood vessel dependent. Likewise, upon comparison of metastatic burden in the lungs of tumor-bearing SSIL2R and SS.BN3IL2R rats, we observed decreased metastatic lung nodules (Fig. 3E) corresponding to a 7-fold decrease in metastatic burden in SS.BN3IL2R lungs (41 ± 16 RLUs/mg; P < 0.05) compared with SSIL2R lungs (300 ± 161 RLUs/mg; Fig. 3F). Moreover, H&E staining revealed histologically detectable metastatic lesions in 80% of SSIL2R lungs, but only 20% of SS.BN3IL2R lungs examined (data not shown). Collectively, these data suggest that genetic variants on BN chromosome 3 increase tumor BVD, however, with a paradoxical decrease in hematogenous metastasis that is potentially due to decreased vascular invasion.

In most cases, BVD is positively correlated with tumor growth and hematogenous metastasis (33), prompting us to validate whether the paradoxical angiogenesis phenotype seen in SS.BN3IL2R (Fig. 3B) is present in the unmodified parental SS and SS.BN3 consomic strains using two independent methods: a Matrigel plug angiogenesis assay (34) and reanalysis of the DMBA-inducible mammary tumors from Adamovic and colleagues (16). For the Matrigel plug angiogenesis assay, female SS and SS.BN3 consomic rats were implanted with 500 μL of Matrigel supplemented with 500 ng/mL of recombinant rat VEGF164. At 5 days postimplantation, Matrigel plugs were excised and quantified for CD31+ blood vessels across the entire cross section of the plug. Compared with SS plugs (14 ± 3 vessels/mm²; n = 9 rats), the BVD in SS.BN3 consomic rats was approximately 2-fold higher (23 ± 4 vessels/mm²; P < 0.05; n = 19 rats; Fig. 4A and B). Likewise, BVD of SS.BN3 mammary tumors (n = 9) from the DMBA-inducible model showed an approximately 2-fold (P < 0.01) increase relative to SS tumors (n = 9) from the same study (Fig. 4C and D; ref. 16), collectively demonstrating that SS.BN3 has increased angiogenic potential (i.e., the ability to form new blood vessels), despite having decreased tumor growth, vascular invasion, and hematogenous metastasis (Figs. 2 and 3).

Tumor lymphangiogenesis and lymphogenous metastasis

To assess LVD, 231Luc+ tumors that were orthotopically implanted in MFPs of SSIIL2R and SS.BN3IL2R rats were excised at 24 days postimplantation and stained with antibodies against the lymphatic vessel marker, LYVE-1 (Fig. 4A; ref. 35). In contrast to tumor BVD, we observed no difference in LVD between SSIIL2R (41 ± 7 vessels per field; n = 5 rats) and SS.BN3IL2R
Cancer Research
Cancer Res; 74(22) November 15, 2014

The density of SH2B3 and SS.BN3 (chromosome 3) were previously reported in independent F2– and metastasis (Figs. 2 and 3). Overlapping cancer QTLs on rat chromosome 3 affect tumor growth, vascularity, vascular invasion, and lymph node metastasis (Fig. 4), our data suggest that the causative variants on BN chromosome 3 are vascular cell-type specific.

**Sequence analysis of rat chromosome 3**

Our data demonstrate that genetic elements on BN chromosome 3 affect tumor growth, vascularity, vascular invasion, and metastasis (Figs. 2–5). Overlapping cancer QTLs on rat chromosome 3 were previously reported in independent F2 crosses using cancer resistant (BN/SeNHsd and COP/Crl) and cancer susceptible (F344/NHsd and ACI/SegHsd) strains, including the Epm3 QTL (chr3:50.5-88.6 Mb, BN/SeNHsd x F344/NHsd; ref. 17), the Emca5 QTL (chr3:41.0-171.0 Mb, BN/SeNHsd x ACI/SegHsd; ref. 18), and the Ept2 QTL (chr3:44.6-110.2 Mb, ACI/SegHsd x COP/Crl; ref. 19; Fig. 6). Analysis of the F344.BN3 congenic rat (F344.BN-Edpm3BN) showed decreased tumor susceptibility and increased density of malformed tumor blood vessels (32), which closely resembles the SS.BN3IL2Ry consomic phenotypes (Figs. 2 and 3). As the protective alleles in the F344.BN-Edpm3BN QTL (17, 32) and the SS.BN3IL2Ry consomic are BN-derived, the causative alternative alleles in this region are likely shared by SS and F344. Because the causative alleles in the Emca5 QTL (18) and Ept2 QTL (19) are ACI-derived, the protective alternative alleles in this region are likely shared by BN and Cop. Combining all data, we extended the cosegregating sequence analysis to BN and Cop (protective) versus SS, F344, and ACI (susceptible).

We analyzed the distribution of alleles on rat chromosome 3 using WGS of BN/NHsdMcwi, ACI/Eur, Cop/Crl, F344/N, and SS/JrHsdMcwi that was recently generated in collaboration with Atanur and colleagues (27) (Fig. 6A). To identify cosegregating and unique genomic regions that could influence cancer susceptibility, we plotted the total unique and common variants per Mb that cosegregated between two groups: BN/NHsdMcwi and Cop/Crl (tumor-resistant) versus SS, F344, and ACI (susceptible).

**Figure 4.** Increased angiogenic potential in SS.BN3 consomic rats. A, density of CD31+ blood vessels in Matrigel plugs implanted in SS (n = 20) and SS.BN3 (n = 10) consomic rats. B, mean CD31+ blood vessel density in Matrigel plugs implanted in SS and SS.BN3 consomic rats was calculated from the entire Matrigel plug cross-section. Data, the mean vascular density per mm² ± SEM. ***, P < 0.005, as determined by the unpaired Student t test. C, density of SH2B3+ blood vessels in DMB-induced tumors of SS (n = 9) and SS.BN3 (n = 9) consomic rats. D, mean density of SH2B3+ blood vessels was calculated from ×400 images of the entire tumor cross-section that were acquired using an automated microscope. Data, the mean vascular density per area ± SEM. ***, P < 0.001, as determined by the unpaired Student t test.

(41 ± 5 vessels per field; n = 6 rats) tumors (Fig. 4B), which coincided with no differences in metastatic burden in the axillary lymph nodes of either strain (Fig. 4C). Both strains showed a high incidence of lymphatic vessel invasion by 231Luc cells in 80% (SSIL2Rg) and 67% (SS.BN3IL2Rg) of tumors examined (data not shown), indicating a similar propensity of vascular density per mm² calculated from the entire Matrigel plug cross-section. Data, the mean vascular density per mm² ± SEM. For B and C, statistical analysis of data was performed by the unpaired Student t test.

**Figure 5.** Characterization of tumor-associated lymphatic vessels and lymphogenous metastasis in 231Luc+ tumors implanted in SS.BN3IL2Rg and SSIL2Rg rats at 24 days postimplantation. A, visualization of tumor-associated lymphatic vessels using anti-LYVE-1 staining of 231Luc+ tumors implanted in SS.BN3IL2Rg and SSIL2Rg rats. Scale bar, 100 μm. B, mean lymphatic vessel density in 231Luc+ tumors implanted in SS.BN3IL2Rg and SSIL2Rg rats was calculated from three images per tumor (n = 5–6 per strain) acquired at ×100 magnification. Data, the mean vascular density per ×100 field ± SEM. C, lymphogenous metastatic burden was measured by luciferase activity normalized to total milligrams of protein in axillary lymph node lysates from SS.BN3IL2Rg (n = 5) and SSIL2Rg (n = 8) rats. Metastatic burden of individual rats is represented by the dots, and the black bars indicate the average metastatic burden per strain. For B and C, statistical analysis of data was performed by the unpaired Student t test.
ACI/SegHsd, and SS/JrHsdMcwi (tumor-susceptible; Fig. 6B). The overlapping QTL regions (chr3:50.5-88.0 Mb) were significantly enriched by 30-fold (P < 0.001) for cosegregating alleles compared to the average of the rest of chromosome 3 (Fig. 6A–C), indicating that the genetic mechanisms underlying these similar QTLs are likely shared in these regions. Combined, these data suggest that common heritable elements are most likely enriched in the overlapping cancer QTLs (16–19).

Using the cosegregating variant analysis we preliminarily reduced the list of candidate variants from 455,272 total variants on rat chromosome 3 to 15,031 variants (3.3% of total chromosome 3 variants) in the cosegregating region (chr3:50.5–88.0 Mb) that overlapped with the three cancer QTLs (17–19). Of the 15,031 cosegregating variants, 46 lie within coding regions of conserved genes, 10 are expected to cause nonsynonymous amino acid changes, and 2 of these were predicted to be damaging by Provean (http://provean.jcvi.org/seq_submit.php; Table 1 and Supplementary Table S3).

**Comparative genomics**

Our data suggest that cosegregating genetic elements on rat chromosome 3 affect tumor growth, angiogenesis, vascular invasion, and metastasis (Figs. 2–5A). The 37.5-Mb region (chr3:50.5–88.0 Mb) of overlapping cancer QTLs contains 184 conserved and validated genes (Supplementary Table S2) and is syntenic to two regions on human chromosomes 2 and 11 (Fig. 6D) and one region on mouse chromosome 2 (data not show). A total of four genes [**METAP1D** (5), **CDCA7** (5), **PTPRJ** (36), and **CD44** (37–40)] in syntenic regions of the human genome have been associated with breast cancer risk (Fig. 6D), whereas no mouse studies have reported associations for breast cancer in the syntenic mouse region. Of the human risk genes, **METAP1D** and **CDCA7** were associated with breast cancer incidence by genome-wide association study (5). **CD44** has been associated with multiple aspects of human breast cancer risk: incidence (39), age of onset (37, 40), angiogenesis (41), metastasis (42), and survival (39, 43, 44). **PTPRJ** mutations were associated with breast cancer risk by loss of heterozygosity analysis (36) and **PTPRJ** has also been implicated in angiogenesis (45). Strikingly, **PTPRJ** mutation caused disorganized vascular plexus formation in mouse embryos (46), which was marked by increased density of poorly formed blood vessels that are reminiscent of the vascular phenotypes.

Figure 6. Sequence analysis of cosegregating alleles and comparative mapping of rat chromosome 3. A, Venn diagram of sequence comparison of the entire rat chromosome 3 between variants shared by the cancer-resistant BN/NHsdMcwi and Cop/Crl strains versus the cancer-susceptible strains ACI/Eur, F344/N, and SS/JrHsdMcwi. B, density mapping of variants shared by the cancer-resistant BN/NHsdMcwi and Cop/Crl strains versus the cancer-susceptible strains ACI/Eur, F344/N, and SS/JrHsdMcwi. Data, the number of variants per 1-Mb bin. Note the highest density of cosegregating variants overlap with three breast cancer QTLs (labeled brackets). C, Venn diagram of sequence comparison of the shared QTL region of chromosome 3 (50.5–88.0 Mb) between variants shared by the cancer-resistant BN/NHsdMcwi and Cop/Crl strains versus the cancer-susceptible strains ACI/Eur, F344/N, and SS/JrHsdMcwi. D, schematic representation of the overlapping cancer QTLs on rat chromosome 3 and the orthologous regions in human. The overlapping human breast cancer loci are indicated.
Table 1. Cosegregating nonsynonymous variants in the overlapping cancer QTL on rat chromosome 3

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<td>G</td>
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<td>–0.020</td>
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NOTE: Provean prediction of > 2.5 or higher (emboldened) indicates that the amino acid change is likely damaging to protein function.

Abbreviation: aa, amino acid.

Discussion

The heritability of breast cancer is well established (1–3), yet the majority of causative variants remain unknown (5). Of the few known variants, most are considered tumor cell-autonomous (47–49), with far less emphasis placed on germline variants that might affect breast cancer progression through the tumor microenvironment. We developed an experimental model that specifically focused on mapping genetic variants underlying differences in the tumor microenvironment. CXM utilizes genetically "fixed" tumor cells that are xenografted into rat strains with different genetic backgrounds, so that any observed differences in tumor progression (e.g., growth, vasculogenesis, and metastasis) would be attributable to the tumor microenvironment, including interaction between the tumor and host. From a clinical perspective, these data show that germline variants in the tumor microenvironment affect breast cancer risk and should be taken into account in future studies. As a technical advancement, this work demonstrates that CXM can be used to directly map germline variants in the tumor microenvironment for the first time.

Using CXM, we demonstrated that genetic variants on BN chromosome 3 inhibited tumor growth and lung metastasis of 231\textsuperscript{1}\textsuperscript{lac} breast cancer cells (Figs. 2 and 3), without affecting lymph node metastasis (Fig. 5). Sequence analysis by cross-strain comparisons and comparative analysis prioritized a list of 6 candidate genes for future study (Fig. 6; Table 1). These 6 candidate genes were prioritized using functional predictions of nonsynonymous variants (Fsipl2 and Pramel7) and shared association with human breast cancer [METAPD (5), CDC47 (5), PTPRJ (36), and CD44 (37–40)]. Within the region, it is also possible that other nonsynonymous variants and intergenic variants have functional consequences beyond the limitations of prediction software. Thus, validating these candidates genes will likely require further narrowing of the QTL by congenics (26, 50, 51), gene expression analysis, and/or directly testing candidates by gene editing (20, 52, 53), all of which we have done previously for other disease loci (see below for description).

Breast cancer risk QTL on rat chromosome 3

Previously, we (16) and others (17–19) identified overlapping cancer QTL on rat chromosome 3 (50.5–88.0 Mb). A congenic of the Edpm3 QTL (F344.BN-Edpm3BN) indicated that the protective cancer QTL is likely due to malformed immature blood vasculature, despite the tumors having a higher BVD (32). Similarly, we observed significant tumor growth inhibition in the SS.BN3IL2R consomic compared with SSIL2R\textsupersomic (Fig. 2); with a paradoxical increase in tumor-associated blood vessels (Fig. 3A and B), suggesting that the vascular phenotypes underlying the rat chromosome 3 QTL are likely derived from the tumor microenvironment. Tumor metastasis in the previous rat QTL studies was not reported (17–19, 32), as most inducible rat mammary tumors are poorly metastatic. By CXM, we were able to show that variants on BN chromosome 3 also affect vascular invasion and distal metastasis of 231\textsuperscript{1}\textsuperscript{lac} cells (Figs. 3 and 5). Despite the increased BVD in SS.BN3IL2R\textsupersomic, both vascular invasion of tumor cells and hematogenous metastatic burden were significantly lower in SS.BN3IL2R\textsupersomic (Fig. 3C–E), suggesting a potential mechanism that attenuates metastasis by limiting invasion or transport of tumor cells. Collectively, CXM extended the previous findings (17–19) by demonstrating that (i) genetic variants in the rat chromosome 3 QTL likely act through the tumor microenvironment; (ii) the genetic mechanisms are blood vessel specific; and (iii) tumor growth and hematogenous metastasis are also attenuated by the genetic variants.

Blood vasculature is essential for tumor growth and metastatic progression (34). Our data suggest that germline variants affecting tumor-associated blood vessels can significantly affect cancer risk (Figs. 2 and 3). Vascular defects also decrease risk of solid tumors in Down syndrome patients with trisomy 21, due to increased gene dosage of DSCR1 (55). The important...
distinction is that Down syndrome is a non-mendelian genetic disease, whereas our findings suggest that common germline variants could have similar effects in a much broader spectrum of patients. Our data also suggest that "looks can be deceiving," i.e., metastatic potential and tumor growth are frequently correlated with vascular density (33), but based on our findings, this might not always be the case. Instead, we propose that variants affecting vascular function and maturity will similarly affect breast cancer progression, which might be overlooked as a prognostic indicator by relying on vascular density only. Finally, from a translational viewpoint, the findings of this study indicate that some germline variants could potentially function as "genetic switches" between hematogenous and lymphogenous metastasis. Thus, a better understanding of germline variants that affect tumor metastasis will likely yield better patient stratification for vascular-specific adjuvant therapies [e.g., bevacizumab (56), TKi (57), etc.].

Using CXM and similar strategies to localize cancer risk variants

From a technical standpoint, we posit that CXM can be tailored to further elucidate causative variants using additional mapping strategies (e.g., F2 intercross, congeneric, gene editing, etc.) or to test interactions with other clinically relevant subsets of breast cancer (e.g., luminal, lobular, inflammatory, etc.). Our rationale for first using a consomic was (i) to develop the general technique and (ii) to localize the phenotype to a specific chromosome before proceeding further. Using CXM and comparative mapping, we narrowed the list of potential candidate variants in the rat chromosome 3 QTL by 96.7%. Now, it would be foreseeable to physically narrow the QTL with the ultimate goal of localizing the causative variant. Because TALENs and ZFNs do not require specific rat strains (we have now gene edited on >10 backgrounds: http://rgd.mcw.edu/wg/physgenknockouts), it would be possible to generate SCID rats (or mice) on any background. For example, one could reduce the SS.BN3 cancer QTL by selectively backcrossing SS.BN311.2Ry to SS11.2Ry until the region of BN chromosome 3 has been sufficiently narrowed to resolve the causative variant, followed by expression analysis and TALEN- or ZFN-mediated gene editing of candidate genes on the minimal SS.BN311.2Ry congeneric background, an approach that we have used previously for other disease loci (53). Finally, although we developed CXM for the rat, we have also demonstrated the utility of TALEN- or ZFN-mediated gene editing in the mouse (58) and therefore, CXM could be used in mice and potentially other model organisms.

Experimental considerations of the CXM strategy

There are several limitations of cancer xenograft models that should be considered when interpreting CXM data. First, like all xenograft models, CXM utilizes SCID rodents that lack fully intact immune systems and thus genetic variants that affect tumor immunity might not be accessible. Second, xenotransplantation of human cancer cells into rodent backgrounds might not recapitulate species-specific mechanisms, but rather only mechanisms conserved across species. Finally, some germline variants likely affect both malignant tumor cells and the nonmalignant stromal cells, whereas CXM would only detect effects on the nonmalignant stroma. Despite these limitations, there are also considerable advantages of CXM, including the ability to use etiologically defined and clinically relevant human cell lines that are transgenically tagged for better tracking in vivo.

Perspective

CXM is the first model for mapping germline variants in the tumor microenvironment. To do so, CXM combines genome editing (via TALENs), a genetic mapping tool (the consomic), and a widely used model of human breast cancer (the tumor xenograft). As proof-of-concept, we demonstrated that CXM can detect germline variants in an established breast cancer QTL (Figs. 2 and 3; refs. 17–19). Using CXM, we also demonstrated that the causative variants on rat chromosome 3 affected the tumor blood vasculature, which potentially disrupted tumor growth and metastasis. Follow-up studies using cell culture systems, localization of candidate gene expression, and tissue-specific gene editing will be required to determine whether the causative variants function directly or indirectly through the blood vasculature. In summary, CXM is a technical advance that localizes cancer phenotypes to a single chromosome and could be used to isolate causative variants/genes by congeneric mapping and/or candidate gene targeting.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.J. Flister, B.T. Endres, N. Rudemiller, A. Lemke, I. Roy, A.M. Geurts, C. Moreno, P.E. North, M.B. Dwinell, J.D. Shall
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.J. Flister, B.T. Endres, N. Rudemiller, A.B. Sarkis, S. Santarriaga, S.-W. Tsaih, J.D. Pons, P.E. North, P.S. LaViolette
Writing, review, and/or revision of the manuscript: M.J. Flister, N. Rudemiller, A.B. Sarkis, C. Moreno, Z. Lazarova, J. Lazar, J.D. Shall, R. Roy, P.S. LaViolette, M.B. Dwinell, J.D. Shall, H.J. Jacob
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Study supervision: J. Lazar, H.J. Jacob

Acknowledgments

The authors thank M. Teisch, E. Christenson, C. Duris, M. Tschannek, J. Wendt-Andrae, M. Grzybowski, S. Kalloway, J. Foeckler, and R. Schilling for excellent technical support.

Grant Support

This study was supported by an Interdisciplinary Cancer Research Fellowship from the Medical College of Wisconsin Cancer Center (M.J. Flister), a Froedtert Hospital Foundation Skin Cancer Center Research Award (Z. Lazar). NCI grant R01CA77876 (J.D. Shall), and NHLBI grants 5R01HL055168 and R24HL1147401A1 (H.J. Jacob). M.J. Flister is also supported by an NHLBI training grant (ST32HL007790). A.M. Geurts is supported by an NIH Director’s New Innovator Award (DP2OD008396).

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Received November 12, 2013; revised July 8, 2014; accepted July 9, 2014; published OnlineFirst August 29, 2014.
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CXM: A New Tool for Mapping Breast Cancer Risk in the Tumor Microenvironment

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