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Gene expression and epigenetic profiles of mammary gland tissue: Insight into the differential predisposition of four rat strains to mammary gland cancer

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ABSTRACT

Rats are excellent experimental models for studying breast cancer, but rat strains differ in susceptibility. Among the four strains used in this study, Fischer rats are less susceptible to spontaneous breast cancer, yet they are highly prone to extremely severe metastatic and drug-resistant tumors, in those case where they actually develop the disease. In contrast, Sprague Dawley rats are the most susceptible to spontaneous breast cancer among the strains. ACI rats are highly prone to estrogen-induced cancer. Long–Evans rats are commonly used in mammary gland carcinogenesis studies. The molecular mechanisms of differential breast cancer susceptibility among rat strains are not well understood. Here, gene expression analysis was conducted in the mammary gland tissue of four rat strains – August × Copenhagen Irish (ACI), Long Evans, Fischer-344 and Sprague Dawley – to evaluate possible explanations for the differing breast cancer predispositions. According to the DAVID functional annotation analysis, there were at least eleven, five, and one significantly different pathways, respectively, in Fischer-344, Long–Evans and Sprague Dawley rats, in comparison to ACI rats. Two strains, Fischer-344 and Long–Evans, displayed differential expression in the complement and coagulation cascades, chemokine signaling, PPAR signaling, renin–angiotensin system, ECM-receptor interaction, focal adhesion and glutathione metabolism pathways. The only pathway that was significantly different between the Sprague Dawley and the ACI rats was the ribosome pathway. Our data indicate that general cancer susceptibility and predisposition to the development of aggressive and metastatic cancer are independent genetic conditions. Moreover, we have identified several important differences in the basal epigenetic profile of four rat strains with varying degrees of susceptibility to spontaneous and induced mammary carcinogenesis.

1. Introduction

Breast cancer is a complex disease, with over one million cases diagnosed worldwide each year and a predicted 50% increase in cancer rates by the year 2020 [1]. Many studies have focused on genetic factors contributing to cancer susceptibility; rodent models have been widely used [2–4]. Rodent strains have been bred that are either resistant or susceptible to specific carcinogens. For example, the ACI rat, which exhibits high sensitivity to elevated levels of estrogen, with a remarkably reduced latency of tumor development, is used extensively in studies of estrogen-induced mammary cancer [5–7]. In comparison, Copenhagen (COP) and Fischer-344 rats are relatively resistant to estrogen-induced mammary carcinogenesis and can be used to provide insight into protective factors [7–9]. ACI rats have a very low level of spontaneous and radiation-induced mammary tumors, while the majority of female Fischer-344, Long–Evans and Sprague Dawley rats are more prone to these tumors, suggesting that cancer susceptibility involves a complex array of predetermining factors [5,9–18]. Szpirer and Szpirer described the susceptibility of various strains to carcinogen- and hormone-induced mammary cancer and to spontaneous mammary cancer [19]. The Sprague Dawley rat strain was shown to be susceptible to chemical- and radiation-induced breast cancer but resistant to cancer induced by hormones. When analyzing the karyotypes of rat strains with different susceptibility to

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mammary cancer, Rees et al. [20] did not find any strong correlation between karyotype and cancer susceptibility, except for the Long–Evans and Fischer rats.

Genetic differences have been explored through genome sequencing, shedding light on genetic determinants of mammary cancer susceptibility. Recent publications suggest that a possible mechanism of estrogen-induced mammary cancer in the ACI rat is mediated via estrogen-induced mammary cancer loci (EMCA) [5,6,21,22]. Interestingly, several rat strains that were investigated have Emca loci, which influence the latency and the number of tumors; however, these loci are often expressed in a different manner [6,21]. Candidate genes within the Emca loci include DNA methyltransferases, suggesting a role of epigenetics in determining mammary cancer susceptibility (http://www.ensembl.org/). Further evidence of epigenetic regulation of susceptibility was shown in Wistar–Kyoto and SHR/y rats, which showed different expression patterns of renin and angiotensinogen [23], differences thought to be regulated by epigenetic landscaping. As the body of evidence linking epigenetic alterations with the genesis of cancer grows, the focus has shifted toward elucidating the underlying epigenetic phenotype that predisposes individuals to genomic instability and cancer. Epigenetic malfunctions may cause abnormal tissue differentiation, loss of cell adhesion, migration of abnormal cells and up-regulation of estrogen receptor pathways [24].

The epigenome is primarily composed of two interconnected dynamic processes by which mammalian cells can modify expression of their genomes without altering the DNA sequence: DNA methylation and covalent histone modifications [25]. The best studied of these mechanisms is DNA methylation, which has been shown to be both tissue- and species-specific [26–29]. Numerous studies have identified DNA methylation as a mechanism for regulating cell processes, including genomic imprinting and X-chromosome inactivation, acting as a repressor of gene expression to maintain genome stability [28,30–34] Histone modifications (methylation (me), acetylation (ac), phosphorylation (ph), ubiquitination (ub) or sumoylation (su) of amino acid residues in histone H1, H2A, H2B, H3 and H4 tails), on the other hand, can lead to either transcriptional activation or repression, depending on the type. These modifications are thought to occur in a hierarchical fashion, with present markers at the same or nearby sites, which influence further posttranslational modifications [35–39]. Some studies have shown, for example, that acetylation of histone H3 lysine 9 (acH3K9) or lysine 14 (acH3K14), which is associated with a more relaxed chromatin structure, is able to restrict histone 3 lysine 9 methylation (meH3K9), a repressive modification [40]. The goal of this study was to identify differences in the genetic and epigenetic background of four commonly used laboratory strains, female ACI, Long–Evans, Fischer-344 and Sprague Dawley [7–9,18,41,42], that may account for differences in spontaneous mammary tumors.

2. Methods and materials

2.1. Animal models and tissue preparation

Five-week-old intact female Long–Evans rats were obtained from Charles River (Wilmington, MA). Female Fischer-344, Sprague Dawley and ACI rats from Harlan Sprague Dawley, Inc. were similarly conditioned (Indianapolis, IN). The animals were housed two per cage in a temperature-controlled (24 °C) room in a 12 h light-dark cycle and given ad libitum access to water and an NIH-31 pelleted diet. Each strain group consisted of five animals that were humanely sacrificed at 6 weeks of age without any previous treatment.

The paired caudal inguinal mammary glands were excised. One gland was frozen immediately in liquid nitrogen and stored at −80 °C for subsequent analyses. The contralateral gland was fixed in 4% PFA for 48 h, processed and embedded in paraffin. Tissue microarrays, 4 μm thick, were constructed from 4.5-mm representative cores from each rat and mounted on positively charged slides for immunohistochemical (IHC) analysis.

2.2. RNA isolation

Total RNA was isolated using the Illustra RNAspin mini kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Mammary gland tissue (50–70 mg) was processed following the manufacturer's instructions. Samples were eluted in Ultrapure DNase/RNase-free distilled water, provided in the kit. RNA samples were quantified by ultraviolet spectroscopy (NanoDrop, Wilmington, DE) and were further assessed for RNA integrity (RIN) on the Aglient 2100 Bioanalyzer (Santa Clara, CA) using the RNA Nano-chip Kit. RNA samples with RIN values of 7 or better followed through to analysis.

2.3. Whole-genome gene expression profiling

2.3.1. Library preparation

cRNA was created using Ambion's Illumina TotalPrep RNA Amplification Kit (Applied Biosystems, Carlsbad, CA) with an input of 500 ng total RNA per sample. Briefly, oligo-dT primers were used to synthesize first-strand cDNA containing a phage T7 promoter sequence. The single-stranded cDNA was converted into a double-stranded DNA template via DNA polymerase. RNase H acted simultaneously to degrade any RNA that was not converted to cDNA, and the samples were purified in filter cartridges to remove excess RNA, primers, enzymes and salts. The recovered cDNA was subjected to in vitro transcription, using biotinylated UTPs. This step created labeled and amplified cRNA. A final purification step removed unincorporated NTPs, salts, inorganic phosphates, and enzymes to prepare samples for hybridization.

2.3.2. Hybridization and detection

Illumina's direct hybridization assay kit was used to process samples according to the manufacturer's protocol (Illumina, San Diego, CA). Briefly, 750 ng from each cRNA sample was hybridized to the Illumina Rat-Ref-12 Whole Genome Expression BeadChip arrays overnight. Afterward, a10-min incubation with supplied wash buffer at 55 °C preceded a 5-min room-temperature wash. The arrays were incubated in 100% ethanol for 10 min. A second room temperature wash for 2 min with gentle shaking completed this high stringency wash step. The arrays were blocked with buffer for 10 min and washed before a 10-min probing with steptavidin-Cy3 (1:1000). After a 5-min wash at room temperature, BeadChips were dried and imaged. Six controls were also built into the Whole-Genome Gene Expression Direct Hybridization Assay system to cover aspects of the array experiments. These included controls for the biological specimen (14 probes for housekeeping controls), three controls for hybridization (six probes for Cy3-labeled hybridization, four probes for low stringency hybridization, and one probe for high stringency hybridization), signal generation (two probes for biotin control) and ~800 probes for negative controls on an eight-sample BeadChip. Arrays were scanned on the iScan platform (Illumina) and data were normalized and scrutinized in Illumina BeadStudio software.

2.3.3. BeadChip statistical analysis and data processing

The false discovery rate (FDR) was controlled by the Benjamini–Hochberg method. The Illumina Custom Model took the FDR into account and was used to analyze the data. Differential gene expression (at least 0.5-fold change) from sham treated animals was determined to be statistically significant if the p-value after adjustment with the Benjamini–Hochberg method was less than 0.05. Values were transformed to show a log2 scale.

Lists of regulated transcripts were put into the web-based DAVID Bioinformatics Resources 6.7 (NIAID/NIH) Functional Annotation Tool [43,44]. This program was used to group genes into functionally relevant categories and pathways for further analysis of association of the genetic profiles with breast cancer susceptibility. The minimum number of genes in each altered pathway has been set to three. The pathways were deemed significantly altered if at least 80% of the genes were shifting the pathway in the same direction [45].

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time PCR was performed to confirm the Whole-Genome Gene Expression results for regulation direction (either up or down) of select genes. Six genes (*Olah1*, *Ancra2*, *Col3a*, *Per2*, *Serpinb6a* and *Rpl30*) were selected from the gene list of significantly differentially expressed transcripts, representing only a preliminary review of the acquired gene expression data. *β-Actin* was used as a reference gene. All reactions were performed using cDNA synthesized from the same RNA extraction as the BeadChip experiments, using 500 ng of sample for the Bio-Rad iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Samples were stored at −20 °C for long-term storage and at 4 °C until used for subsequent qRT-PCR reactions.

Primers were designed using the NCBI database and PrimerQuest (Integrated DNA Technologies, Inc., Coralville, IA). Primers were as follows: *Olah1* forward primer 5′-ATC ACC TTC TGG ATT TCG GAG GCA-3′ and reverse primer 5′-AGA GAA GAG CTT TGG AGG GCT TGT-3′; *β-Actin* reference gene forward primer 5′-CCT CTG AAC CCT AAG GCC AA-3′ and reverse primer 5′-AGC CTG GAT GGC TAC GTA CA-3′; *Ancra2* forward primer 5′-TCT CTG TCT GTT CAC CAG TTG GCA-3′ and reverse primer 5′-TCT ACC ACA GCT ATT TGC CCG TGT-3′; *Col3a* forward primer 5′-ATG TCC TTG ATG