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MicroRNA 'signature' during estrogen-mediated mammary carcinogenesis and its reversal by ellagic acid intervention

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ABSTRACT

Dysregulated miRNA expression has been associated with the development and progression of cancers, including breast cancer. The role of estrogen (E_2) in regulation of cell proliferation and breast carcinogenesis is well-known. Recent reports have associated several miRNAs with estrogen receptors in breast cancers. Investigation of the regulatory role of miRNAs is critical for understanding the effect of E_2 in human breast cancer, as well as developing strategies for cancer chemoprevention. In the present study we used the well-established ACI rat model that develops mammary tumors upon E_2 exposure and identified a 'signature' of 33 significantly modulated miRNAs during the process of mammary tumorigenesis. Several of these miRNAs were altered as early as 3 weeks after initial E_2 treatment and their modulation persisted throughout the mammary carcinogenesis process, suggesting that these molecular changes are early events. Furthermore, ellagic acid, which inhibited E_2-induced mammary tumorigenesis in our previous study, reversed the dysregulation of miR-375, miR-206, miR-182, miR-122, miR-127 and miR-183 detected with E_2 treatment and modulated their target proteins (ERα, cyclin D1, RASD1, FoxO3a, FoxO1, cyclin G1, Bcl-w and Bcl-2). This is the first systematic study examining the changes in miRNA expression associated with E_2 treatment in ACI rats as early as 3 week until tumor time point. The effect of a chemopreventive agent, ellagic acid in reversing miRNAs modulated during E_2-mediated mammary tumorigenesis is also established. These observations provide mechanistic insights into the new molecular events behind the chemopreventive action of ellagic acid and treatment of breast cancer.

1. Introduction

Breast cancer is the most commonly diagnosed cancer among women in the United States and is the second leading cause of cancer deaths among women [1]. The development of breast cancer has been associated with numerous risk factors including genetic, environmental, hormonal, and dietary influences [2,3]. Despite all of the available data on breast cancer risk, the etiology of the majority of human breast cancers is still not readily identifiable. Accumulating data from epidemiological studies, experimental animal models, and cell culture studies have shown that reproductive hormones, particularly estrogens, play a critical role in breast cancer etiology. Nearly 70% of breast tumors express estrogen receptor α (ERα) [4]. Up-regulated ERα during early stages of tumorigenesis has been identified as an important factor in stimulating proliferation of mammary cells leading to tumor development [5]. Other breast cancer risk factors, such as age at menarche, postmenopausal obesity, and hormone replacement therapy, are believed to increase risk by increasing estrogens.

MicroRNAs (miRNAs) are endogenous small noncoding RNAs of 20–25-nucleotide that are involved in post-transcriptional control of gene expression. miRNAs regulate gene expression by binding to 3′ untranslated region (UTR) of their target mRNAs causing degradation or translational silencing of targeted transcripts [6]. Recent studies documented aberrant miRNA expression profiles in breast cancer compared with normal breast tissues, with tumor suppressors miRNAs miR-10b, -125b and -145 down-regulated and oncogenic miRNAs miR-21 and miR-155 up-regulated [7]. Moreover, miRNA signatures were reported to reliably predict breast cancer biopathological features, such as ER, PR and HER2/neu receptor status [8]. Kovalchuk et al. suggested deregulation of several cellular epigenetic processes such as alterations in DNA methylation, histone modifications, and aberrant miRNA expression play crucial

Abbreviations: E_2, 17β-estradiol; miRNA, microRNA; ERα, estrogen receptor α; PR, progesterone receptor; ACI rat, August-Copenhagen Irish rats; EA, ellagic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; cDNA, complementary deoxynucleic acid; IPA, ingenuity pathway analysis; CCND1, cyclin D1; RASD1, RAS dexamethasone-induced 1; FOXO, Forkhead box O; CCNG1, cyclin G1 and Bcl-2, B-cell lymphoma 2.

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role in the mechanism of E_2-induced mammary carcinogenesis in ACI rats, especially in the tumor initiation process [9].

Since the role of miRNAs in progression of cancer is evident, targeting miRNAs has been considered as a viable treatment option. Accumulating experimental evidence has shown that over-expression or knock-out of specific miRNAs could inhibit cancer cell proliferation and regression of tumors in animal models [10–12]. Recent reports have also indicated the ability of pharmacological agents and dietary components to modulate the alterations of miRNA expression induced by carcinogens [13–15]. Ellagic acid (EA) is a plant polyphenol abundant in raspberries, pomegranates, and walnuts [16,17]. In other experimental cancer models, EA was reported to decrease the incidence of chemically-induced lung [18], mammary [19], small intestinal [20], colon [21] and oral [22] tumors. In previous work we demonstrated the cancer preventive effects of dietary intervention using EA against E_2-mediated mammary tumors in the ACI rat model [23–25].

The goals of this study were to: (i) measure the changes in expression of miRNAs during the course of E_2 treatment in the rat mammary tissue; (ii) delineate canonical/functional pathways regulated by genes that are potential targets of the modulated miRNAs; (iii) analyze effect of EA intervention on the E_2-modulated miRNAs and, (iv) determine the expression pattern of select targets at mRNA and protein levels.

2. Materials and methods

2.1. Tissue samples

Female August-Copenhagen Irish (ACI) rats (Harlan Sprague–Dawley, Inc., Indianapolis, IN) were provided AIN 93M diet and water ad *libitum*. Non-tumor (distal normal mammary) and tumor tissues were obtained from two E_2-induced mammary tumorigenesis studies: Study 1 in which female ACI rats were treated without (control) or with continuous low doses of E_2 and euthanized at 3, 12 (close to the appearance of first palpable mammary tumor) and 26 (when tumor incidence reaches nearly 90%) weeks. Study 2 [24] in which female ACI rats were treated without (control) or with continuous low doses of E_2 alone, EA alone or E_2 in combination with EA and euthanized after 8, 16 and 28 weeks. E_2 was administered via subcutaneous silastic implants (1.2 cm, 9 mg E_2), while EA was administered via the diet (400 ppm). Tissues from these studies were stored at −80 °C. For initial microarray studies, archived tissues from the Study 1 were utilized, while tissues from Study 2 were used to investigate the effect of EA intervention on miRNA modulation. Animal experimentation protocol was approved from the Institutional Animal Care and Use Committee (IACUC) at University of Louisville.

2.2. Isolation of RNA

*mir*Vana miRNA Isolation kit (Applied Biosystems) was used to isolate total RNA for microarray analysis and mRNA expression studies. Small RNA was further enriched from total RNA for qPCR analysis of miRNAs according to the manufacturer's protocol. Trace genomic DNA in the crude total RNA samples was removed by incubation with 10 units of DNase I per 100 μg RNA (Ambion, Austin, TX) at 37 °C for 30 min. The concentration of the total and small RNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA integrity was verified with a Bioanalyzer 2100 (Agilent, Palo Alto, CA).

2.3. miRNA expression profiling microarray

Total RNA containing low molecular weight RNA was hybridized to a GeneChip® miRNA Array (Affymetrix, Santa Clara, CA) at 48 °C and 60 rpm for 16 h, then washed and stained on Fluidics Station 450 (Fluidics script FS450_0003) and finally scanned on a GeneChip® Scanner 3000 7G using Affymetrix Command Console 1.0 (Affymetrix, Santa Clara, CA). Microarray signals were analyzed and heat maps were generated using Partek Genomic Suite 6.5 RMA algorithm. ANOVA and Correlation analyses (Pearson's Pairwise Comparison and Spearman's rank correlation) were performed and FDR reports were generated using Partek Pro Software (Partek, St. Charles, MO). Following the identification of differentially expressed miRNAs, the predicted targets for these miRNAs were identified using TargetScan 6.2 data base. Functional profiling and canonical pathway analysis was carried out by uploading the data set of predicted targets for the significantly altered miRNAs to the Ingenuity Pathways Analysis (IPA).

2.4. qRT-PCR validation of miRNA microarray data

Molecules demonstrating consistent modulation at 2 or more time points in microarray observations were selected for qRT-PCR validation. For analysis of the 11 selected miRNAs (miR-122, -127, -142–5p, -182, -183, -205, -206, -25, -335, -375 and -429), the individual TaqMan human MicroRNA Assays were used. Briefly, 25 ng of total RNA was reverse-transcribed in a final volume of 20 μl with 12.5 nM of each RT primer using the TaqMan MicroRNA Reverse Transcription Kit. TaqMan miRNA PCR kit was used to perform PCR reactions on the ABI 7900 Real-Time PCR System (Applied Biosystems, Foster City, CA). The reactions were initiated in a 96-well optical plate at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative miRNA expression was assessed using the differences in normalized Ct (ΔΔCt) method after normalization to 5S rRNA. Fold changes were calculated by $2^{-\Delta\Delta Ct}$.

2.5. qRT-PCR for target gene expression

One-Step SYBR green qRT-PCR Kit (Quanta Biosciences, Gaithersburg, MD) was used to perform cDNA synthesis and PCR amplification simultaneously from 100 ng of total RNA according to the manufacturer's instructions. Reactions were run under the following conditions: hold at 50 °C for 10 min, 95 °C for 5 min, then 40 cycles at 95 °C for 10 s and 60 °C for 30 s. Relative gene expression was assessed using the differences in normalized Ct (ΔΔCt) method after normalization to 18S rRNA. Fold changes were calculated by $2^{-\Delta\Delta Ct}$.

2.6. Immunoblot analysis

The mammary tissue lysates (40 μg) were resolved on SDS–polyacrylamide gel and immunoblotted with various primary antibodies as previously described [26], ERα(alpha) (Estrogen receptor α) (Proteintech, Chicago, IL), Bcl-2, CCNG1 (cyclin G1) (Santa Cruz Biotechnology, Santa Cruz CA), RASD1 (RAS dexamethasone-induced 1), Bcl-w, FoxO3a (Forkhead box O3a), FoxO1 (Forkhead box O1), (Cell Signaling, Danvers, MA) and CCND1 (cyclin D1) (Abcam Cambridge, MA) were purchased. Appropriate secondary antibody was used and detection carried out using enhanced chemiluminescence (Thermo Scientific, Waltham, MA). Equal loading of the proteins was confirmed by staining the polyacrylamide gel with coomassie blue stain.

3. Results

3.1. miRNA expression profile during E_2-mediated mammary tumorigenesis

The effect of E_2 on miRNA expression in non-tumor and tumor tissues of ACI rats was analyzed using microarray for the expression levels of 351 rat specific miRNAs. Principal component analysis (PCA) was performed on 28 samples (3 normal mammary samples each from 3 and 12 weeks untreated (control) and E_2-treated groups; 5 samples of non-tumor (distal normal mammary) and 6 tumors tissues from E_2-treated group and 5 from untreated group at the 26 week time point) and view the global changes of miRNA expression induced by E_2 treatment at different treatment time points. The normalized Ct values of 351 miRNAs were used for this analysis and the results for the first 3 principal components are shown in Fig. 1a. The samples could be roughly divided into 2 groups. Untreated (control) samples at 3, 12 and 26 weeks are grouped together while E_2-treated samples including the non-tumor and tumor tissues are clustered together. Within the E_2-treated group, animals showed a much higher level of variation than the control, which may be caused by larger interindividual responses to the E_2 treatment. The PCA results suggest that the miRNA expression was globally altered by E_2 treatment at 3, 12 and 26 weeks. We performed statistical comparison between the groups of samples (combining $a \geqslant 1.5$-fold change threshold and ANOVA $p < 0.05$). Unsupervised hierarchical clustering analysis of the positively detected (Ct < 35) miRNAs is shown in Supplementary Fig. S1. We identified 33 miRNAs that were significantly different between the untreated control and E_2-treated groups at two or more time points. Clustering of the samples was performed for a second time using the 33 significantly modulated miRNAs. The resulting dendrogram could be divided into two clearly separated sections: a first section containing the untreated control samples