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miRNAs Differentially Expressed in Prostate Cancer Cell Lines after Soy Treatment

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Abstract. *Background: MicroRNAs (miRNAs) are small non-coding RNAs that have aberrant expression in prostate cancer tissues. miRNAs are involved in the initiation and progression of cancer, and several miRNAs have been characterized as tumor suppressors or oncogenes. It has been shown that some miRNAs can be directly regulated from their own promoters by epigenetic alterations in cancer cells. Moreover, phytoestrogens are known to have epigenetic action on gene transcription. Hence, we conducted here an examination of the miRNA expression profile in human prostate cancer cell lines after soy phytoestrogen treatment. Materials and Methods: The comparative miRNA expression profiles of prostate cell lines (PC-3, DU145, LNCaP) after a 48-h treatment of 40 μM genistein, 110 μM daidzein, or 2 μM 5-azacytidine (5-AZA, a demethylating agent) were conducted with a Taqman low-density array. Results: We found that out of 377 miRNAs tested, 180, 170 and 150 miRNAs were amplified with 2% of variation in the triplicate in PC-3, DU145 and LNCap cells, respectively, and only 5 miRNAs for PC-3 and DU145 cells and 4 miRNAs for LNCap exhibited a significant change in their expression. Treatment with genistein or daidzein had similar effects on miRNA regulation to those of 5-AZA treatment. Conclusion: This work demonstrated a new role of isoflavones on the regulation of miRNAs in prostate cancer.*

MicroRNAs (miRNAs) belong to a class of small non-coding RNAs that regulate the expression of protein-coding genes (1, 2). MicroRNAs are 21 to 23 nucleotides long and control gene expression by binding to complementary sites in the 3'-untranslated regions (3'-UTRs) of target mRNAs, triggering either translational inhibition or mRNA degradation (3). However, miRNAs can also positively regulate gene expression by binding to partial complementary sequences in the promoter regions of genes (4). Recent studies have shown the aberrant expression of miRNAs in prostate cancer (5-8). Dahiya's team also reported that genistein, a natural, nontoxic dietary isoflavone, and trichostatin A (TSA), a histone deacetylase inhibitor and potent anticancer drug, alone or in combination significantly down-regulated the expression of the minichromosome maintenance (MCM) gene family in both androgen-dependent LNCaP and androgen-independent PC3 cells (9). Moreover, this team also reported that after combination treatments with genistein, 5-azacytidine (5-AZA), a demethylating agent, and TSA, there was an increase in the expression of miR-145, suggesting that silencing of miR-145 occurs through DNA methylation in prostate cancer cell lines (10).

Thus, this study was designed to examine the miRNA expression profile in prostate cancer cell lines by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) with Taqman low-density arrays through DNA methylation after treatment with genistein, daidzein and 5-AZA.

Materials and Methods

Cell lines and culture. The human prostate cancer cell lines DU145, PC-3 and LNCaP were obtained from the American Type Culture Collection (Manassas, VA, USA). DU145 is known to be negative for androgen receptor (AR), as is the PC-3 cell line and LNCaP cells are positive for AR. DU145 cells were cultured in Eagle's minimum essential medium (EMEM), PC-3 cells in F-12K medium, and LNCaP cells in RPMI-1640. All cultures were supplemented

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with 1% glutamine (Sigma®), 0.1% gentamycin (Sigma®, St Louis, MO, USA) and 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA, USA) and were grown in a humidified atmosphere at 37˚C containing 5% CO_2.

Cell treatments. Genistein (4',5,7-trihydroxyisoflavone; Sigma®) and daidzein (4',7-dihydroxyisoflavone; Sigma®) were solubilized in dimethyl sulfoxide (DMSO) at respective concentrations of 40 μM and 110 μM, determined previously by flow cytometric analysis as corresponding to causing cell cycle arrest in G_2/M (11). 5-AZA (Sigma®), a DNA methyltransferase (DNMT) inhibitor, was solubilized in phosphate-buffered saline (PBS) at a concentration of 2 μM. All treatments were performed for 48 h.

RNA extraction. RNA was isolated from 10^6 cells using *mir*Vana™ miRNA isolation kit (Ambion, Austin, TX, USA). Recovered RNA was quantified using a Nanodrop 8000 spectrophotometer (Nanodrop Technology®, Cambridge, UK) and RNA integrity was assessed using a 2100 Bioanalyser (Agilent, Palo Alto, CA, USA). RNA extracts with RNA integrity number values >9 were included for further analysis.

Reverse transcription of miRNAs with stem-loop primers. Total RNA (1 μg) was used for cDNA preparation using specific stem-loop primers (12) according to the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems Incorporation, Foster City, CA, USA).

Quantitative real-time PCR using low-density TaqMan miRNA array. A predesigned array called miRNA panel A (366 TaqMan® miRNA expression assay preconfigured in a 384-well format, microfluidic cards; Applied Biosystems), were used for real-time PCR with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The miRNA array in this study was configured into 377 miRNAs. A total of 100 μl reaction mixture with 50 μl cDNA template (100 ng) and an equal volume of TaqMan® universal master mix (Applied Biosystems) was added to each line of array after gentle vortex mixing. Thermal cycler conditions were as follows: 2 min at 50˚C, 10 min at 94.5˚C and 30 s at 97˚C, and 1 min at 59.7˚C for 40 cycles. SDS 2.2 software (Applied Biosystems) was used to analyze and normalize the RT-qPCR data (13). In addition, fold changes in gene expression in treated cells normalized to an endogenous reference gene (MammU6-4395470) and relative to the normalized expression in untreated cells were calculated. Array experiments were performed in triplicate using samples from three independent biological experiments. All data are expressed as the mean±SD. Differences between groups were calculated with Student's *t*-test. A *p*-value <0.05 was defined as being statistically significant.

Results

Regulation of miRNAs in prostate cancer cell lines by isoflavones and 5-AZA. We analyzed expressions of 377 mature miRNAs by Q-RT PCR using miRNA microfluidic cards (Applied Biosystems) in prostate cancer cell lines (PC-3, DU145, LNCaP) after a 48-h treatment of 40 μM genistein, 110 μM daidzein, or 2 μM 5-AZA. In PC-3 cells, out of 377 miRNAs tested, 180 miRNAs were amplified, with 2% variation in the triplicate, and only five miRNAs exhibited a significant change in their expression (Figure 1A). Expression of four miRNAs was down-regulated and only one miRNA was up-regulated by more than 3-fold in PC3 cells compared to untreated cells. Expression of four miRNAs, namely miR-125a, -125b, -15b and -320 significantly decreased ($p \leq 0.01$) in PC3 cells after treatment with genistein, daidzein, or 5-AZA by comparison to untreated cells. Expression of one miRNA, namely miR-548b-5p, was significantly increased ($p \leq 0.01$) in PC3 cells after treatment with genistein, daidzein, or 5-AZA by comparison to untreated cells. The trend of effect was the same with the two isoflavones, genistein and daidzein as with the demethylating agent 5-AZA for specific miRNAs. In DU145 cells, out of 377 miRNAs tested, 170 miRNAs were amplified, with 2% of variation in the triplicate, and only five miRNAs exhibited a significant change in their expression. Expression of five miRNAs was down-regulated by more than 3 to 5-fold in DU145 cells compared to untreated cells. Expression of five miRNAs namely miR-155, -208b, -211, -376a and -411 were significantly decreased ($p \leq 0.01$) in DU145 cells after treatment with genistein, daidzein, or 5-AZA by comparison to untreated cells. Expression was reduced with the two isoflavones, genistein and daidzein similar to 5-AZA. In LNCaP cells, out of 377 miRNAs tested, 150 miRNAs were amplified with 2% of variation in the triplicate and only four miRNAs exhibited a significant change in their expression. Expression of three miRNAs was down-regulated by more than 2-fold for two miRNAs and only one miRNA was up-regulated in LNCaP cells compared to untreated cells. Expression of 3 miRNAs namely miR-494, -520g, -542 was significantly reduced ($p \leq 0.01$) in LNCaP cells after treatment with genistein, daidzein, or 5-AZA by comparison to untreated cells. Expression of one miRNA, namely miR-15a, was significantly increased ($p \leq 0.01$) in LNCaP cells after treatment with genistein, daidzein, or 5-AZA by comparison to untreated cells. The trend of regulation for each miRNA was the same with the two isoflavones, genistein, daidzein as with the demethylating agent (5-AZA).

Validated targets of the altered miRNA expressions by isoflavones and 5-AZA in prostate cancer cell lines. Validated targets were compiled from miRecords database (14) and miR2Disease database (15), downloaded from http://mirecords.biolead.org/ and http://mir2disease.org/. For PC3 cells, down-regulated miRNAs namely miR-125a, miR-125b and miR-15b, have respectively 3, 43 and 2 validated targets each. On the contrary, there are no validated targets for the down-regulated miR-320 and the up-regulated miR-548b-5p. Two targets, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (*ERBB2*) and v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)

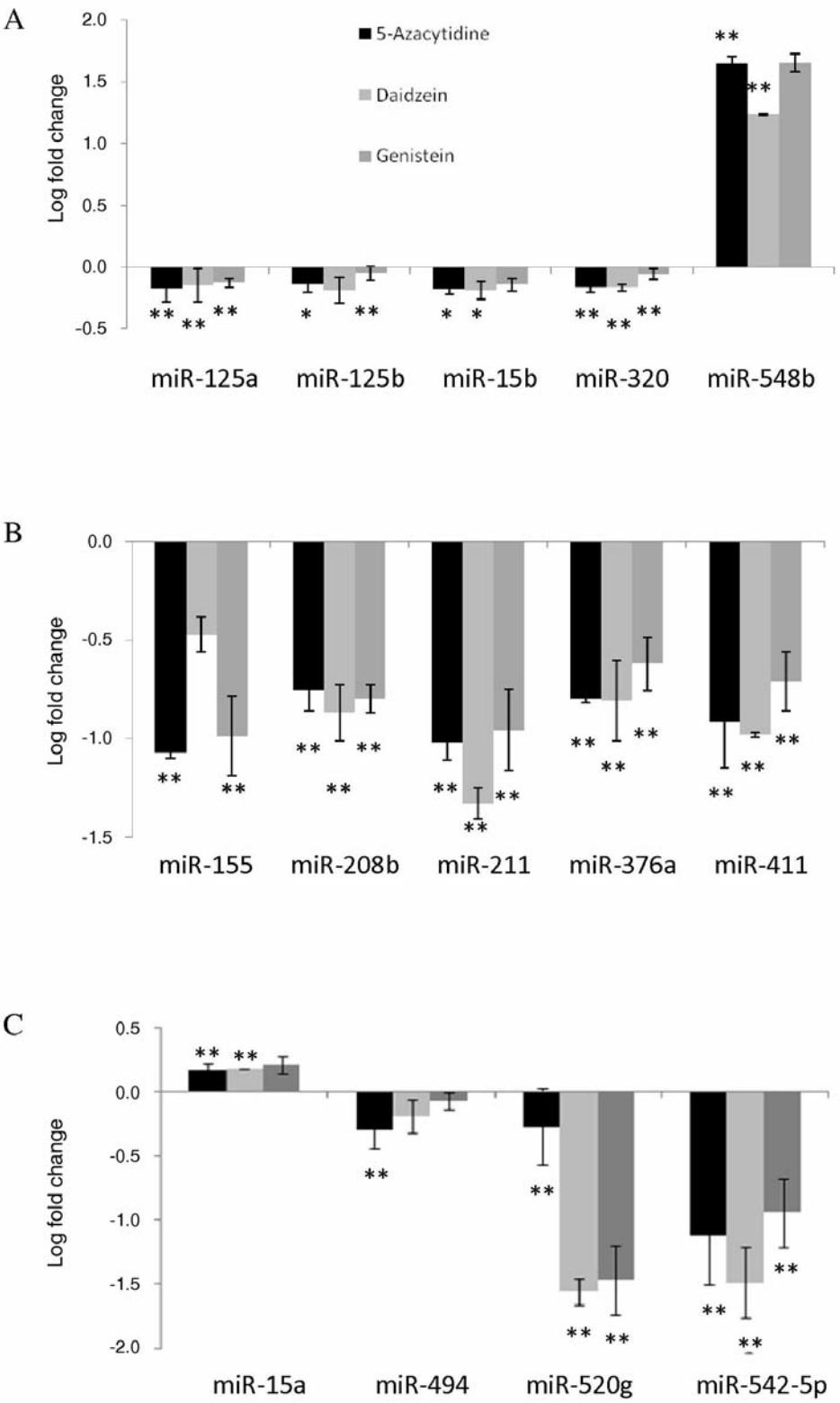

Figure 1. *miRNA profiling in PC3 (A), DU145 (B), LNCap (C) cell models after 2 μM 5-azacytidine (demethylating agent), 110 μM daidzein and 40 μM genistein treatment. miRNA profiling using Q-RT PCR for mature miRNAs using stem-loop miRNA primers in treated and untreated cell lines. Fold changes (log2) are shown with respect to untreated cells. Error bars represent the standard deviation. Statistical significance: *p≤0.05; **p≤0.01.*

(*ERBB3*), were found in interaction with the down-regulated miRNA, namely miR-125a and miR-125b. For DU145 cells, down-regulated miRNAs, namely miR-155 and miR-376a, have respectively 10 and 3 validated targets, but without any interactive targets. On the contrary, there are no validated targets for the other down-regulated miRNAs, miR-208b, miR-211 and miR-411. For LNCap cells, treated by isoflavone, the up-regulated miRNA, namely miR-15a, has 65 validated targets. The down-regulated miRNA, namely miR-520g, has 1 validated target. There are no validated targets for the down-regulated miRNAs miR-494 and miR-542-5p. An interaction between miR-15a and miR-520g was found with the target gene vascular endothelial growth factor (*VEGFα*).

Discussion

In the present communication we showed that out of 377 miRNAs tested, 180, 170 and 150 miRNAs, respectively, were amplified with 2% of variation in the triplicate in PC-3, DU145 and LNCap cells; of these, 5 miRNAs for PC-3 and DU145 cells and 4 miRNAs for LNCap exhibited a significant change in their expression. The phytoestrogen treatment, compared with the therapeutic agent (5-AZA) demonstrated an action on the DNA methylation because genistein and daidzein exhibited modification in their expression similar to 5-AZA. Increased DNA methylation of the CpG islands in the promoter region of genes is well established as being a common epigenetic mechanism for the silencing of tumor suppressor genes in cancer cells (16). Epigenetic silencing of a gene can be reversed by drugs, such as 5-AZA, which form a covalent complex with the active site of methyltransferase resulting in generalized demethylation.

Epidemiologic studies suggest that intake of a soy-rich diet may have a protective effect against prostate cancer (17, 18). Prostate cancer incidence is lower in Asian men who are high soy consumers as compared to Westerners, who consume low amounts (19). Genistein and daidzein, the two principal soy isoflavones, show a wide array of chemopreventive actions (20). The anticancer effects of genistein and daidzein have been described to involve several signalling pathways and mechanisms that lead to cell cycle arrest, apoptosis, invasion, metastasis and angiogenesis (11, 21). Genistein is a naturally occurring isoflavonoid that is abundant in soy products and has been identified as an inhibitor of protein tyrosine kinases and thus it has a key role in cell growth and apoptosis (22). It has also been reported to have estrogenic properties and neoplastic activity in multiple tumor types (23). It was also found to have epigenetic effects in the mouse prostate (24) and in prostate cell lines by regulating miR-1296 (9, 10). The above findings prompted us to examine isoflavone effects on the expression of a large group of miRNAs. They play an important role in various biological an metabolic processes, including differentiation, signal transduction, cell maintenance, disease (2, 25) and cancer (5, 26). Bioinformatics predictions indicate that miRNAs regulate about 30% of all protein-coding genes (27). Moreover, the role of 5-AZA in the reversal of epigenetic silencing of genes prompted us to compare its effects with those of genistein and daidzein. Some findings suggest that epigenetic changes can control the expression of tumor suppressor intronic miRNAs by directly controlling their host genes. This reveals an additional mechanism and anticancer effect of epigenetic therapy (7). This work improves our understanding of the mechanism(s) by which miRNAs are modified by soy isoflavones and the effects of epigenetic drugs (5-AZA) in cancer.

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