Androgen receptor decreases CMYC and KRAS expression by upregulating let-7a expression in ER$^-$, PR$^-$, AR$^+$ breast cancer

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Abstract. It is generally known that the decision to use anti-estrogen therapy is based on the expression of estrogen and progesterone receptors in breast cancers. Recent studies have shown that androgen receptor (AR) is frequently expressed in ER$^-$, PR$^-$ breast cancer and plays an important role in the prognosis of breast cancer patients. Furthermore, AR can increase the global expression of microRNAs, post-transcriptional gene regulators that play a crucial role in the initiation and progression of breast cancer. In this study, we investigated the functions and relations of AR, related miRNAs and target proteins in ER$^-$, PR$^-$, AR$^+$ breast cancer. The results showed that androgen-induced AR activating signal directly upregulates let-7a expression, downregulates CMYC and KRAS protein expression, and inhibits cell proliferation in ER$^-$, PR$^-$, AR$^+$ breast cancer cells. Overexpression of let-7a inhibits cell proliferation and downregulates CMYC and KRAS protein expression, whereas inhibition of let-7a expression by specific antisense oligonucleotides increases cell growth and upregulates CMYC and KRAS protein expression. We performed *in situ* hybridization for let-7a and immunohistochemical staining for CMYC and KRAS using sequential sections obtained from surgically-resected breast cancer tissues and observed an inverse correlation between the staining pattern of let-7a and its target proteins. Androgen-induced AR activating signal upregulates let-7a that targets CMYC and KRAS and contributes to ER$^-$, PR$^-$, AR$^+$ breast cancer pathogenesis. Elucidation of this pathway will help develop new therapies.

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Introduction

Breast cancer is a sex steroid hormone-responsive tumor; therefore sex steroid hormones and their receptors play a pivotal role in the cell growth and progression of this type of tumor. Since the 1950's, there has been an enormous number of reports on estrogen, estrogen receptor (ER), ER blockade, aromatization inhibition and related topics that has been accompanied by great progress in mainstream clinical management of breast cancer (1). However, androgen and androgen receptor (AR) have been clinically and experimentally neglected and so less information is currently known about the role of androgens and AR in breast cancer. To date emerging evidence indicates androgen and AR have a complex and significant function in breast cancer cell growth and tumor progression. Studies have shown that the androgen signaling pathway exerts inhibitory effects on the growth of normal mammary epithelial cells and plays a protective role in the pathogenesis of breast cancer (2-5). Emerging evidence indicates that AR is expressed in breast cancer and may serve as a good prognostic factor (6-10). Because AR expression has also been reported in roughly 50% of patients with estrogen receptor-negative and progesterone receptors-negative (ER$^-$, PR$^-$) breast cancer (11,12), identifying the underlying mechanisms of androgen and AR is important in the design of appropriate therapies for estrogen-insensitive neoplasms. Our previous study (13) showed that among 327 female Chinese cases with invasive ductal breast carcinoma, 72.5% also had detectable AR expression. AR was found in 53.2% of the ER$^-$, PR$^-$ breast cancers in this group. Survival analysis suggested that the patients whose tumors expressed AR had a more favorable prognosis than those whose tumors did not. Similar to our results another study (14) showed that in a population-based study, 77% of invasive breast cancers were AR-positive (AR$^+$) and AR expression was frequent even in molecular subtypes of invasive cancer that are ER$^-$. Further exploration of the role of androgens and AR in breast cancer is essential and will contribute to the development of new therapies for AR$^+$ tumors, especially ER$^-$, PR$^-$, AR$^+$ breast cancer patients who see little or no benefit from anti-estrogen therapy.

Androgens act on target cells by binding to the cognate receptor AR, a member of the nuclear receptor superfamily.

AR is a ligand-dependent transcription factor. Once bound to androgen AR is activated. Activated AR can recognize the androgen response elements (AREs) located at (or close to) the promoter and enhancer region of androgen-dependent genes and then activate the transcription machinery, including that for microRNA (miRNA) transcription (15-17). Recently, several studies have been published that suggest a role for the AR in the transcriptional regulation of miRNA expression (18-20).

MiRNAs are evolutionally conserved approximately 22 nucleotide-long short non-coding RNA molecules that repress target gene expression by binding to complementary sequences found in the 3'-untranslated region (UTRs) of target mRNAs. They participate in diverse biological functions including development, cell proliferation, differentiation, and apoptosis (21-24). Accumulating evidence indicates that miRNA alterations are present in various types of human cancer, including breast cancer, and play a crucial role in the initiation and progression of human cancer through their function as tumor suppressors or oncogenes (25).

The complex relationships among androgens, AR, miRNAs and target mRNAs can be summed up as follows: androgens activate AR by binding to AR and then activated AR can control related miRNA transcription. Processed miRNA can suppress target mRNA expression. In this study, we focused on a representative miRNA in AR activated cells to explore the relationship among AR, this miRNA and its targets in ER^-, PR^-, AR^+ breast cancer. To date, this type of question has not been explored. The results presented here will contribute to the understanding of ER^-, PR^-, AR^+ breast cancer pathogenesis and help design new therapies for estrogen-insensitive neoplasms.

Materials and methods

Cell culture and treatment. MCF-7, MDA-MB-453 and MDA-MB-231 human breast cancer cell lines were chosen because MCF-7 cell line expresses high levels of ER, PR and AR whereas MDA-MB-453 and MDA-MB-231 cell lines express high levels of AR in the absence of ER and PR (26-28). 5α-dihydrotestosterone (DHT, Sigma-Aldrich, MO, USA) was used as it is a non-aromatisable androgen and possesses the highest affinity for AR among natural androgens. All cells were obtained from the American Type Culture Collection (ATCC) and were maintained at 37˚C in a humidified atmosphere containing 5% CO_2 in 75 cm^2 flasks containing minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 mg/ml streptomycin. Cells were passaged every 3-4 days when they reached 80% confluence and were harvested with 0.25% trypsin/EDTA. Before each experiment, cells were grown in phenol red-free (PRF) DMEM, containing 5% charcoal-treated fetal calf serum (PRF-CT), for 3 days and then serum starved in PRF DMEM for 24 h to synchronize the cells. All experiments were performed in 2.5% PRF-CT. Cells were treated with either DHT or vehicle alone at 10^{-8} M. DHT was dissolved in 100% ethanol and added to media immediately prior to use.

MiRNA microarray analysis. The MCF-7 and MDA-MB-453 cell samples were analyzed by KangChen (KangChen Bio-tech Inc.) in miRNA microarray experiments. Total RNA was extracted from MCF-7 and MDA-MB-453 cells that were treated with DHT or vehicle alone using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA quantification and quality assurance were assessed by NanoDrop ND-1000 and RNA integrity and DNA contamination were assessed by denaturing agarose gel electrophoresis. The total RNA was labeled with Cy3 or Cy5 fluorescent dyes by miRCURY™ Array Power Labeling kit (Cat#208032-A, Exiqon) according to the manufacturer's protocols. Each miRCURY LNA microRNA array (v.11.0, Exiqon, Vedbaek, Denmark) was hybridized with a single sample labeled with either Cy3 or Cy5. Gene Pix 4000B scanner and GenePix Pro 6.0 software (Axon Instruments, Union City, CA, USA) were used to scan images. Each group was hybridized with three miRCURY LNA Arrays in triplicate with independent samples for DHT-treated cells or vehicle-treated cells. Background subtraction and normalization were performed. We selected miRNAs whose expression intensities (ForeGround:BackGround) reached at least 1000 and expression levels differed by at least 2-fold between DHT-treated cells and vehicle-treated cells.

Real-time reverse transcription PCR (real-time RT-PCR). ER^-, PR^-, AR^+ MDA-MB-453 and MDA-MB-231 cells were analyzed. To detect the mature miRNA let-7a level, a stem-loop RT-PCR assay was performed (29). Briefly, 2 μg of small RNA was reverse-transcribed to cDNA using M-MLV reverse transcriptase (Promega) with the following primers: let-7a-RT, 5'-GTC GTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGAT ACGACAACTA-3'; and U6-RT, 5'-GTCGTATCCAGTGCA GGGTCCGAGGTATTCGCACTGGATACGACAAAATAT GGAAC-3', which can fold to a stem-loop structure. The specific let-7a cDNA fragment was amplified along with an endogenous control U6 snRNA using the following primers: let-7a-Fwd, 5'-GCCGCTGAGGTAGTAGGTTGTA-3'; U6-Fwd, 5'-TGC GGGTGCTCGCTTCGGCAGC-3', and a universal downstream primer reverse, 5'-CCAGTGCAGGGTCCGAGGT-3'. PCR cycles were as follows: 94˚C for 4 min, followed by 40 cycles of 94˚C for 30 sec, 50˚C for 30 sec, 72˚C for 40 sec. SYBR Premix Ex Taq™ Kit (Takara) was used following the manufacturer's instructions, and the RT-PCR was performed and analyzed by 7300 RT-PCR system (ABI). All primers were synthesized by BGI Inc.

Chromatin immunoprecipitation (ChIP) assay. ChIP assays were performed using MDA-MB-453 cells to determine if activated AR directly upregulates the expression of let-7a (30). Briefly, chromatin DNA was extracted from harvested cells by sonication and precleaned by incubating with normal rabbit serum (IgG) and protein A/G-Sepharose beads. It was then immunoprecipitated with rabbit anti-AR antibody (PG-21, Upstate). The following primers were used: 5'-CAAAGTTTC TAAACGGCTTC (forward) and 5'-AGGATATTTGGTACA CTCTG (reverse) for amplifying the -3922/-3707 fragment and 5'-TTTTACATTGGGCATAGCCG (forward) and 5'-TAG GCATTTGGAAGTTGGAC (reverse) for the -3036/-2786 fragment.

Vector construction. To construct the pcDNA3.1/pri-let-7a expression vector, we first amplified a 203-bp DNA fragment carrying pri-let-7a from genomic DNA using the following PCR primers: let-7a-sense, 5'-CGCGGATCCACTGTGGGATGA

GGTAGTAGGT-3' and let-7a-antisense, 5'-CGCGAATTCTCC AGGCCATAAACAAATGC-3'. The amplified fragment was inserted into the pcDNA3.1 (+) vector at the *Bam*HI and *Eco*RI sites.

Transfection. The vehicle-treated MDA-MB-453 and MDA-MB-231 cells were transfected with vectors and the DHT-treated MDA-MB-453 and MDA-MB-231 cells were transfected with antisense oligonucleotide (ASO) using Lipofectamine 2000 (Invitrogen) at 24 h after plating. Transfection complexes were prepared according to the manufacturer's protocols. The final oligonucleotide concentration was 10^{-9} M, and the final vector concentration was 0.5 mg/l. The transfection medium was replaced at 4 h post-transfection. The oligonucleotides complementary to let-7a were synthesized by IDT (Coralville, IA, USA) and their sequences were as follows: let-7a ASO, 5'-AACTATAC AACCTACTACCTCA-3'; and control ASO, 5'-GTGGATAT TGTTGCCATCA-3'.

Cell proliferation assay. Charcoal-stripped MDA-MB-453 and MDA-MB-231 cells were plated in replicates of 6 at a density of 7000 cells/well in 96-well microtiter plates. At 24 h after seeding, cells were treated with DHT or vehicle alone at 10^{-8} M and reagents were replenished every 3 days. Cell viability and proliferation were measured using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. At days 1-7, the cells exposed to DHT or vehicle were added to MTT. Four hours later, dimethyl sulfoxide (DMSO) was added to each well to dissolve the resulting formazan crystals. After shaking for 20 min, the absorbance at 570 nm was detected using a μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA). MTT assay was also used to measure the viable proliferating cells at days 1-7 after the MDA-MB-453 and MDA-MB-231 cells were transfected.

Flow cytometry analysis. After 48 h, the charcoal-stripped MDA-MB-453 and MDA-MB-231 cells exposed to DHT or vehicle were detached from the plates using trypsin, rinsed with PBS and fixed in 70% (v/v) ethanol. The cells were then rehydrated in PBS and incubated with RNase (100 μg/ml) and propidium iodide (60 μg/ml) (Sigma-Aldrich, MO, USA). Cells were analyzed using the FACS Calibur System (BD Biosciences, San Jose, CA, USA), and the cell cycle phase was determined by Cell Quest analysis. Proliferation index (PI) was calculated as follows: PI = (S+G2/M)/G1 (S, G2/M and G1 refer to the percentage of cells in S phase, G2/M phase and G1 phase, respectively) (31). Flow cytometry analysis was also used to measure cell cycle phase at 48 h after the MDA-MB-453 and MDA-MB-231 cells were transfected.

Western blot analysis. After 48 h the charcoal-stripped MDA-MB-453 and MDA-MB-231 cells exposed to DHT or vehicle were lysed with RIPA lysis buffer and proteins were harvested. All proteins were resolved on 10% SDS denaturing polyacrylamide gel and then transferred onto a nitrocellulose membrane. Membranes were incubated with anti-KRAS, anti-CMYC or anti-GAPDH antibody (Saier Biotech, Tianjin, P.R. China) with blotto overnight at 4˚C. The membranes were washed and incubated with horseradish peroxidase (HRP) conjugated secondary antibody (Saier Biotech). Protein expression was assessed by enhanced chemiluminescence and exposure to chemiluminescent film. Lab Works™ Image Acquisition and Analysis Software (UVP) were used to quantify band intensities.

Patients and tissue samples. Formalin-fixed and paraffin-embedded (FFPE) tissue specimens were obtained from 24 female patients (mean age: 54.5 years, range from 41 to 70 years) who underwent surgical resection for breast cancer from 2003 to 2004 at Tianjin Medical University Cancer Institute and Hospital. All cases were ER^- and PR^- invasive ductal carcinoma (IDC) and all the patients had been treated according to modern guidelines, including the use of adjuvant chemotherapy for IDC and irradiation for lymph node metastasis. The study protocol was approved by the Hospital Human Ethics Committee. Informed consent was obtained from all patients before their surgery and the examination of the specimens.

In situ hybridization (ISH). Locked nucleic acid (LNA) probes complementary to mature let-7a (5'-AACTATACAACCTAC TACCTCA-3') and scrambled negative control (5'-TTCACA ATGCGTTATCGGATGT-3') digoxigenin-labeled at the 5'-position were purchased from Exiqon. Detection of RNAs by ISH utilizing oligonucleotide probes was performed. Briefly, human tissues were deparaffinized, treated with protease (30 min in 2 mg/ml of pepsin), washed in sterile water, and then washed with 100% ethanol and air-dried. Hybridization was performed at 37˚C overnight followed by a low stringency wash in 0.2X SSC and 2% bovine serum albumin at 4˚C for 10 min. The probe-target complex was visualized utilizing a digoxigenin antibody conjugated to alkaline phosphatase acting on the chromogen nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. Nuclear Fast Red served as the counterstain. The slides were scored independently by two pathologists and cases were considered positive if 10% cytoplasmic and/or membranous staining was observed.

Immunohistochemical (IHC) staining. Deparaffinization; endogenous peroxidase inactivation; antigen retrieval of FFPE clinical tissues; and immunostaining with anti-ER (SP1, 1:200 dilution; ZETA), anti-PR (SP2, 1:200 dilution; ZETA), anti-AR (AR441, 1:100 dilution; LabVision), anti-CMYC (SRP00871, 1:200 dilution; Saierbio) and anti-KRAS (SRP01436, 1:200 dilution; Saierbio) antibody were performed as described previously (13). The immunostained sections were evaluated independently by two pathologists in conjunction with the H&E-stained sections from the same lesions. For each antibody, the location of immunoreactivity, percentage of stained cells, and intensity were determined. The evaluation of expression of each protein was determined using the mean of the individual cases. AR, ER, and PR stains were assessed using Allred scores (32). CMYC and KRAS stains were considered positive if 10% of cells showed cytoplasmic and/or membranous staining.

Statistical analysis. The data are reported as the mean ± SD of the values from three independent determinations. Statistical analysis was performed using Student's t-test in comparison with corresponding controls. Associations between ordinal variables were quantified by Spearman rank correlation with Pearson's χ^2 test. Probability values of <0.05 were considered statistically significant. Analyses were run using SPSS17.0.

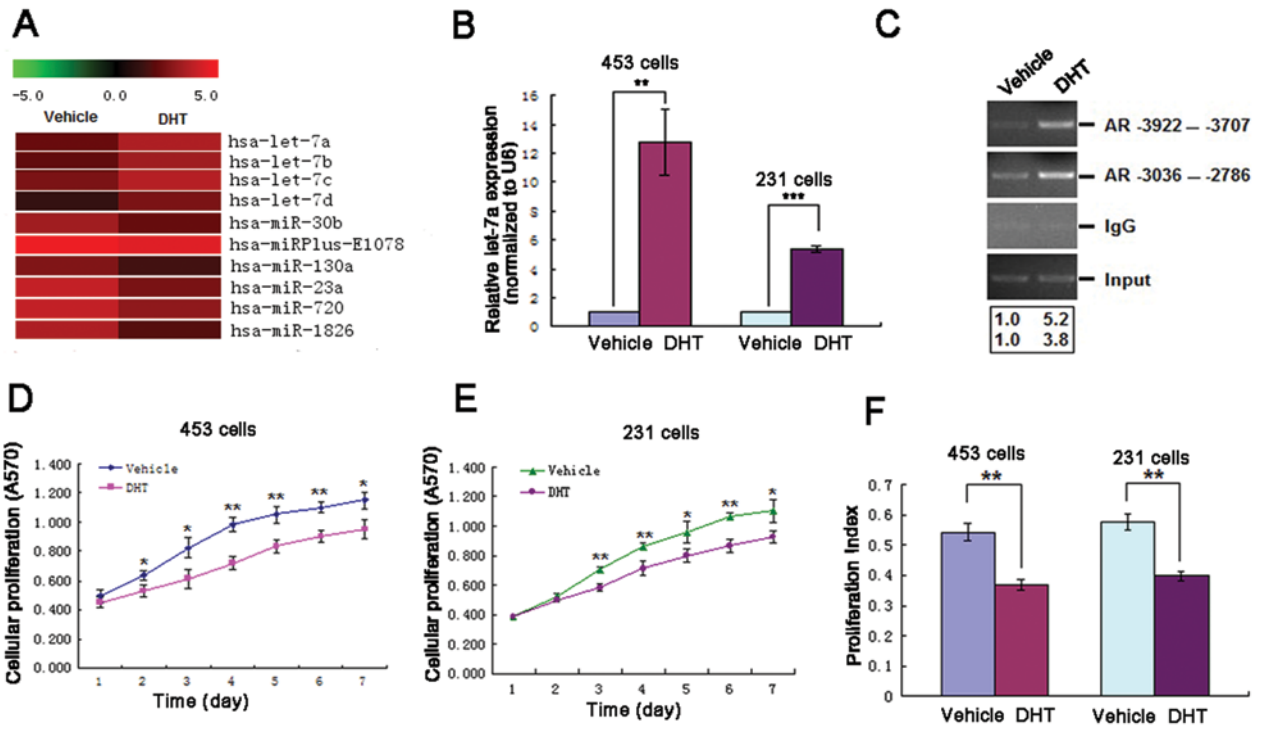

Figure 1. DHT regulates miRNA expression, inhibits cell proliferation and causes cell cycle arrest at the G1-S phases. A, Profiles of miRNAs in DHT-treated and vehicle-treated MDA-MB-453 cells. B, The relative let-7a expression level was detected using real-time RT-PCR assays in MDA-MB-453 and MDA-MB-231 cells. C, ChIP analysis of the AR loading to the 5'-DNA region of let-7a in MDA-MB-453 cells. D, The MTT assay was used to determine relative cellular proliferation in MDA-MB-453 at 1-7 days after treatment with DHT or vehicle. E, The MTT assay was used to determine relative cellular proliferation in MDA-MB-231 cells at 1-7 days after treatment with DHT or vehicle. F, Quantification of the cell cycle phase distribution was analyzed by flow cytometry and the proliferation index (PI) was calculated in MDA-MB-453 and MDA-MB-231 cells. *P<0.05, **P<0.01, ***P<0.000 compared with control.

Results

Profile of miRNAs in DHT-treated and vehicle-treated MCF-7 and MDA-MB-453 cells. To identify critical miRNAs related to androgen-induced AR activating signal in breast cancer, we examined global miRNA expression in DHT-treated and vehicle-treated MCF-7 and MDA-MB-453 cells using the microRNA array (v.11.0, Exiqon) that consists of 847 capture probes for mature human miRNAs. In MDA-MB-453 cells a total number of 10 miRNAs were identified (Fig. 1A) and in MCF-7 cells none was identified using strict criteria that only miRNA undergoing alterations at least 2-fold with expression intensities of at least 1000 were considered as differentially-expressed candidates. Among these differentially-expressed miRNAs in MDA-MB-453 cells, 4 upregulated miRNAs - let-7a, b, c, d were found. Six downregulated miRNAs were identified in the DHT-treated cells when compared with the vehicle-treated cells (Table I). Among them, let-7, as a tumor suppressor miRNA, is reported to be downregulated in many types of solid tumors, including breast cancer (33-35). In our experiments, only let-7a, b, c, d were upregulated in MDA-MB-453 cells. This finding raises the possibility that these upregulated let-7a, b, c, d miRNAs might contribute to the pathogenesis of ER^{-}, PR^{-}, AR^{+} breast cancer. We focused on let-7a and investigated its involvement in ER^{-}, PR^{-}, AR^{+} breast cancer.

Table I. Differentially-expressed miRNAs in MDA-MB-453 cells with at least 2-fold change and >1000 expression intensities.

miRNA[a]	Fold change	Expression intensities (ForeGround-BackGround) DHT[b]-treated group	Vehicle-treated group
hsa-let-7a	2.505687288	2161	909.5
hsa-let-7b	2.297498628	1793	823
hsa-let-7c	2.168932179	2394	1164
hsa-let-7d	2.488421574	1105.5	468.5
hsa-miR-30b	0.457648129	1919.5	833
hsa-miRPlus-E1078	0.498143424	9110.5	4303.5
hsa-miR-130a	0.452923663	1248	536
hsa-miR-29a	0.503380318	3584.5	1711
hsa-miR-23a	0.36632922	3074.5	1068
hsa-miR-720	0.4905976	3047	1417.5
hsa-miR-1826	0.312695862	2281.5	676.5

[a]miRNA, microRNA; [b]DHT, 5α-dihydrotestosterone.

DHT upregulates let-7a inhibiting cell proliferation. In ER^{-}, PR^{-}, AR^{+} MDA-MB-453 and MDA-MB-231 cells the validity of let-7a ectopic expression was confirmed by real-time RT-PCR, which revealed a 13-fold increase in let-7a expression in DHT-treated MDA-MB-453 and a 5-fold increase in MDA-MB-231 cells

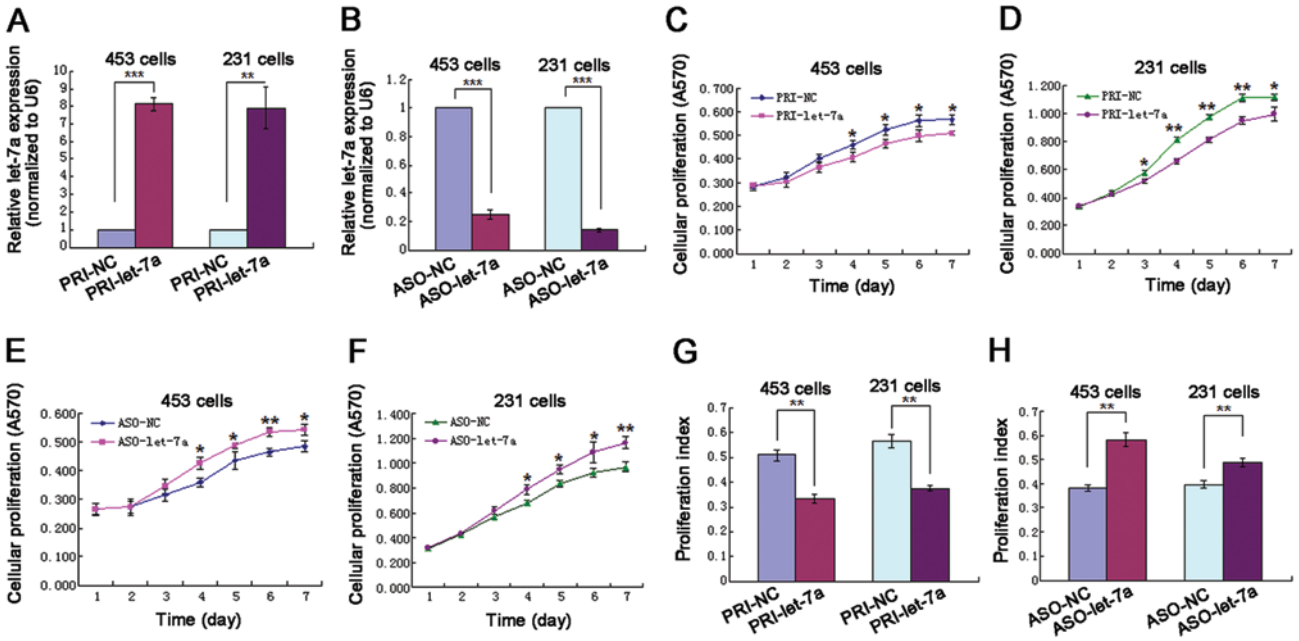

Figure 2. Let-7a inhibits cell proliferation and causes cell cycle arrest at the G1-S phase in MDA-MB-453 and MDA-MB-231 cells. A, In vehicle-treated cells let-7a was overexpressed. B, In DHT-treated cells let-7a was blocked. C-F, The MTT assay was used to determine cellular proliferation at days 1-7 after transfection with vector or ASO. G and H, At 48 h after transfection with vector or ASO quantification of the cell cycle phase distribution was analyzed by flow cytometry and the PI was calculated. *P<0.05, **P<0.01, ***P<0.000 compared with control.

over the vehicle-treated groups (Fig. 1B). To determine if the androgen-induced AR activating signal binds to the 5'-DNA region of the let-7a locus to serve as a transcription factor, we analyzed 4.0 kb in the 5'-region of the let-7a gene and identified a typical TATA box. Using the PROMO 3.0 program 7 potential AREs were identified in the 5'-region of let-7a. To determine AR binding, ChIP was performed, and two primer pairs were used to amplify the -3922/-3707 fragment (corresponding to ARE1) and the -3036/-2786 fragment (corresponding to ARE3). Treatment of MDA-MB-453 cells with 10^{-8} M DHT induced a 5.2-fold increase in AR binding at ARE1 and a 3.8-fold increase at ARE3 (Fig. 1C). Taken together, the results suggest that androgen-AR signaling directly mediates regulation of let-7a. The effects of DHT on MDA-MB-453 and MDA-MB-231 cell proliferation were measured by MTT assay using cells exposed to 10^{-8} M DHT or vehicle for 1-7 days. DHT treatment decreased cell proliferation when compared with vehicle treatment (Fig. 1D and E). Flow cytometric analysis was used to determine the cell cycle distribution of MDA-MB-453 and MDA-MB-231 cells exposed to DHT or vehicle for 48 h. The number of cells in the G1 phase was significantly increased in both types of cells; G1-S arrest was obvious and PI was decreased in the DHT-treated group compared with the vehicle-treated group (Fig. 1F).

The effect of let-7a on the growth of ER^{-}, PR^{-}, AR^{+} breast cancer cells. In order to investigate the biological significance of let-7a in MDA-MB-453 and MDA-MB-231 cells, we used a precursor expression vector (pcDNA3.1/pri-let-7a) to enhance or let-7a ASO to inhibit mature let-7a activity in vehicle-treated or DHT-treated cells. Real-time RT-PCR was used to validate the alteration in let-7a expression level. The let-7a level in the vector-treated group was significantly increased compared with the control group (Fig. 2A), whereas the let-7a level in the let-7a ASO-treated group was significantly decreased compared with the control group (Fig. 2B). These results suggest that the expression of let-7a was successfully altered as designed in our experiments. MTT assay was used to measure the viable proliferating cells at days 1-7 after the MDA-MB-453 and MDA-MB-231 cells were transfected. The MTT data suggests that when let-7a was overexpressed with vector, cell growth was inhibited, an effect similar to that observed with proliferative inhibition by DHT exposure (Fig. 2C and D). When let-7a was blocked with let-7a ASO cell growth activity was elevated, an effect directly opposite to that observed with let-7a overexpression (Fig. 2E and F). Flow cytometry analysis was used to assess the distribution of cells in the cell cycle at 48 h after the MDA-MB-453 and MDA-MB-231 cells were transfected. The data showed that when let-7a was overexpressed the number of cells in the G1 phase was significantly increased, G1-S arrest was obvious and PI was decreased (Fig. 2G), a pattern similar to that seen in cells exposed to DHT. When let-7a was blocked the number of cells in the S phase significantly increased and PI was increased, an effect directly opposite to that observed in cells with let-7a overexpressed (Fig. 2H).

Let-7a negatively regulates CMYC and KRAS at the post-transcriptional level. Translational repression is a major mechanism of miRNA regulation of target gene expression (36). CMYC and KRAS, two oncogenes which are involved in cell proliferation and cell cycle, are known target genes of let-7a (37-40). To confirm the suppressing action of let-7a on CMYC and KRAS the correlation between miRNA and target protein expression was determined. The expression of CMYC and KRAS protein was examined by western blot analysis using MDA-MB-453 and MDA-MB-231 cells that were exposed to 10^{-8} M DHT or vehicle for 48 h when let-7a was upregulated. CMYC and KRAS protein were underexpressed when cells were exposed

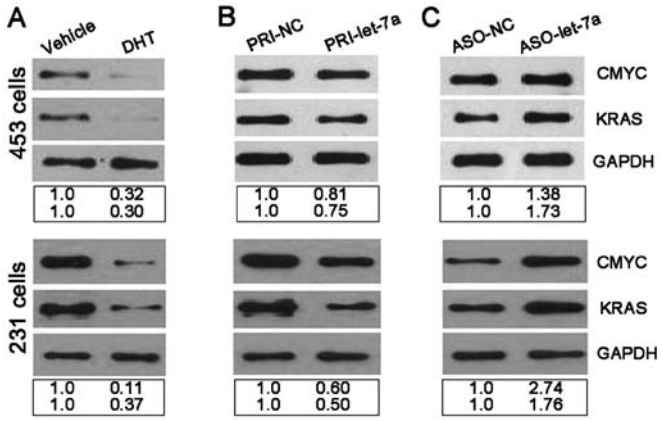

Figure 3. Let-7a downregulates CMYC and KRAS expression in MDA-MB-453 and MDA-MB-231 cells. A, At 48-h of DHT-treatment, the CMYC and KRAS protein levels were assessed by western blot analysis. B and C, At 48 h after transfection with vector or ASO, the expression of CMYC and KRAS protein was examined by western blot analysis. In all the experiments, the expression level of CMYC and KRAS protein in the control group was set to 1 and GAPDH protein was regarded as endogenous normalizer.

to 10^{-8} M DHT (Fig. 3A). Next vehicle-treated MDA-MB-453 and MDA-MB-231 cells were transfected with vector and the expression of CMYC and KRAS protein was examined by western blot analysis. CMYC and KRAS proteins were underexpressed after let-7a was upregulated by vector (Fig. 3B). DHT-treated MDA-MB-453 and MDA-MB-231 cells were then transfected with let-7a ASO and the expression of CMYC and KRAS protein was examined by western blot analysis. The amount of CMYC and KRAS protein was increased after let-7a expression was blocked (Fig. 3C). In conclusion, the above data suggest that let-7a negatively regulates endogenous CMYC and KRAS protein expression through a translational repression mechanism, similar to previous reports (37-40).

The relationship among let-7a, AR, CMYC and KRAS in FFPE breast cancer tissue specimens. In FFPE breast cancer tissue specimens the staining outcome of ISH for let-7a and IHC staining for AR, CMYC and KRAS (Fig. 4; Table II) showed that let-7a expression was negatively correlated with CMYC and KRAS expression. CMYC expression was positively correlated with KRAS expression. No clear correlation was observed between the staining pattern of AR and let-7a, CMYC or KRAS (Table III).

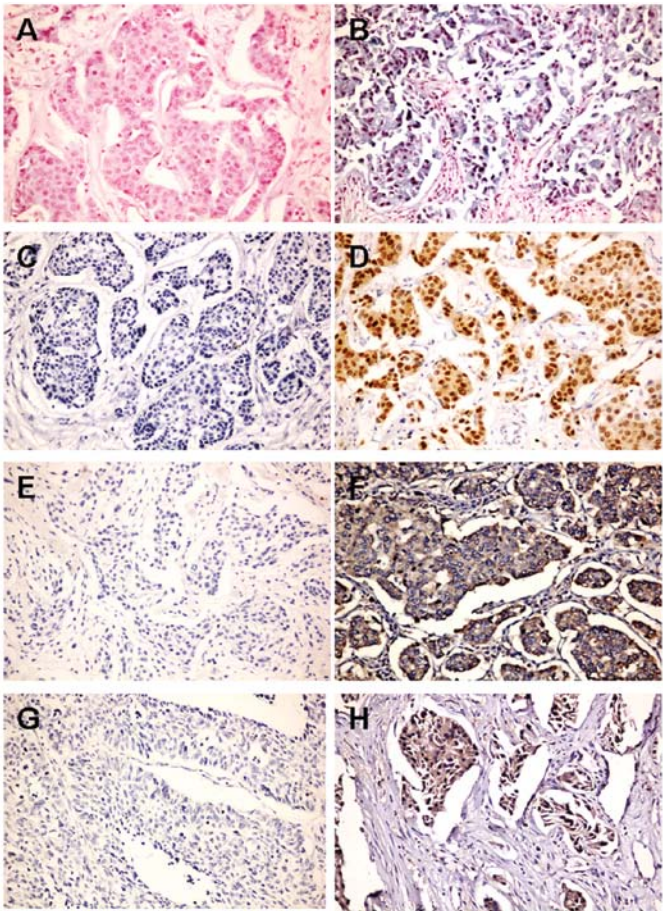

Figure 4. Expression of let-7a by ISH and expression of AR, CMYC and KRAS by IHC in ER^-, PR^-, AR^+ IDC. A, Negative expression of let-7a (x200). B, Positive expression of let-7a (x200). C, Negative expression of AR (x200). D, Positive expression of AR (x200). E, Negative expression of CMYC (x200). F, Positive expression of CMYC (x200). G, Negative expression of KRAS (x200). H, Positive expression of KRAS (x200).

Discussion

Breast cancer is an extraordinarily hormone-dependent tumor. The role of ER and PR are important in regulating cell proliferation and differentiation. Anti-estrogen therapy has therefore been successfully used in treatment of some ER^+ and/or PR^+ breast cancers, yet patients with ER^- and PR^- tumors gain little or no benefit from anti-estrogen therapy. Increasing number of reports show that AR is expressed in a considerable proportion of cases and, of particular interest, AR is also expressed in almost 50% of ER^- and/or PR^- breast cancer (6-14). Identifying the underlying mechanisms of AR are crucial in the design of therapies for estrogen-insensitive neoplasms.

Androgens act on target cells by binding to the cognate receptor AR, a ligand-dependent transcription factor. The AR protein possesses various domains that mediate its different functions (15,16). Unbound AR exists in the cytoplasm as a complex containing several molecular chaperones including Hsp90, Hsp70 and Hsp56. Once bound to a hormone, the AR molecule undergoes a conformational change that results in the shedding of cytosolic heat shock proteins and is translocated into the nucleus where it forms homodimers that associate with nuclear chaperons and coactivators. This homodimer and associated proteins constitute the active form of the receptor that is able to recognize the AREs located at (or close to) the promoter region of androgen-dependent genes. Once bound to AREs the homodimers recruit additional coactivators and activate the transcription machinery, thus increasing specific gene transcription by one or two orders of magnitude (17).

AR can regulate mRNA transcription as well as miRNA biogenesis. Several recent studies describe a role for AR in the transcriptional regulation of global miRNA expression (18-20) based on the observation that androgen-AR signaling directly regulates the expression of certain miRNAs. Recent data indicate that three miRNAs on the miR-125b-2 locus, let-7c, miR-99a and miR-125b-2, are putative AR-regulated targets in prostate cancer cells based on chromatin immunoprecipitation on array analysis (19). In our study, miRNA microarray

Table II. The results of the analysis of ISH and IHC in 24 IDC cases with ER^- and PR^-.

Case	Age	Grade	Let-7a	AR	CMYC	KRAS
1	43	2	+	-	+	+
2	56	2-3	-	-	+	+
3	54	2	-	-	+	+
4	51	2	+	-	+	+
5	57	2	-	-	+	+
6	50	2	+	+	-	+
7	43	2	+	-	+	+
8	63	1-2	+	+	+	-
9	62	2-3	+	+	+	-
10	41	3	-	-	+	+
11	61	2	-	-	+	+
12	44	3	+	-	-	-
13	58	3	+	-	-	+
14	70	3	+	-	+	+
15	51	2	-	-	+	+
16	46	2	-	+	+	+
17	54	1-2	+	+	-	-
18	55	2	-	+	+	+
19	48	3	-	-	+	+
20	60	2	-	-	+	+
21	42	1-2	+	-	+	-
22	67	1-2	-	+	+	+
23	59	2	+	+	-	-
24	56	2-3	+	+	+	+

analysis showed that let-7a, b, c, d were significantly upregulated and real-time RT-PCR analysis revealed a significant increase in let-7a expression in the DHT-treated MDA-MB-453 cells and MDA-MB-231 compared with the vehicle-treated cells. Moreover, ChIP analysis suggests androgen-AR signaling directly upregulates the expression of let-7a.

The discovery of miRNA in the early 1990's has opened a new era of understanding post-transcriptional regulation of genes by small RNAs (41). miRNAs are endogenous, non-coding small RNAs known to repress target gene expression by binding to complementary sequences in the 3'-UTRs of target mRNAs. There are many miRNA changes in different types of human tumors and these miRNAs play an important role in the initiation and progression of tumor as oncogenes and tumor suppressors (42). There are 14 different let-7 family members in mouse and 13 members in human. In human, these different members are let-7a-1, 7a-2, 7a-3, 7b, 7c, 7d, 7e, f7-1, 7f-2, 7g, 7i, mir-98, and mir-202 (43,44). Among these members, let-7a has an identical sequence across various animal species from *Caenorhabditis elegans* to human. Let-7a is widely viewed as a tumor suppressor miRNA because the expression of let-7a is downregulated in many cancer types and during tumor progression (33-35). Sempere *et al* reported that let-7a was decreased in breast cancer cells after performing an ISH assay (45). Yu *et al* found that let-7 regulates key features of breast tumor-initiating cells (BT-IC): self renewal, multipotent differentiation *in vitro*, and the ability to form tumors in NOD/SCID mice (46). Protein expression of the let-7 targets RAS and HMGA2 is high in BT-IC and silenced during differentiation. In our study, 4 upregulated miRNAs: let-7a, b, c, d were identified in the DHT-treated cells as differentially-expressed when compared with the vehicle-treated cells. Expression of let-7a showed a 13-fold increase in MDA-MB-453 cells and a 5-fold increase in MDA-MB-231 cells. To obtain insight into the role of let-7a in the androgen-AR signaling of ER^-, PR^-, AR^+ breast cancer cells, we performed a series of cell function experiments. MTT assay was used to measure the viable, proliferating cells and flow cytometry analysis was used to measure cell cycle phase distribution after the MDA-MB-453 and MDA-MB-231 cells were transfected.

Table III. Correlations between the expression of let-7a, AR, CMYC, KRAS, and two clinicopathological data, expressed as Spearman's ρ with Pearson's χ^2 test for significance.

	Age	Grade	Let-7a	AR	CMYC	KRAS
Age	ρ=1.00					
Grade	ρ=-0.068 P=0.754	ρ=1.00				
Let-7a	ρ=-0.036 P=0.866	ρ=-0.007 P=0.976	ρ=1.00			
AR	ρ=0.305 P=0.147	ρ=-0.362 P=0.082	ρ=0.194 P=0.363	ρ=1.00		
CMYC	ρ=0.045 P=0.836	ρ=-0.080 P=0.711	ρ=-0.472[a] P=0.020	ρ=-0.238 P=0.262	ρ=1.00	
KRAS	ρ=-0.063 P=0.771	ρ=0.255 P=0.230	ρ=-0.531[b] P=0.008	ρ=-0.348 P=0.096	ρ=0.415[a] P=0.044	ρ=1.00

[a]Correlation is significant at the 0.05 level (2-tailed). [b]Correlation is significant at the 0.01 level (2-tailed).

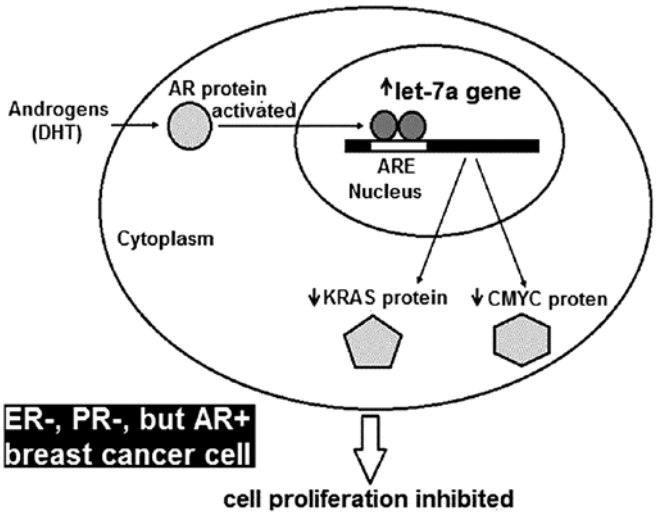

Figure 5. Schematic model for the proposed regulation including DHT, AR, let-7a, CMYC and KRAS.

When let-7a was overexpressed with vector, cell growth was inhibited and an obvious G1-S arrest was observed, similar to the effect seen in cells exposed to DHT. On the contrary, when let-7a was blocked with let-7a ASO cell growth was elevated and the number of cells in the S phase significantly increased.

The oncogenes CMYC and KRAS are known target genes of let-7a that are involved in cell proliferation and cell cycle (34-37). To elucidate the role of let-7a targeting CMYC and KRAS in the process of DHT acting on MDA-MB-453 and MDA-MB-231 breast cancer cells, CMYC and KRAS protein was examined by western blot analysis. The results showed that let-7a negatively regulates endogenous CMYC and KRAS protein expression in both DHT-treated cells and transiently transfected cells.

In FFPE breast cancer tissue specimens the staining outcome of ISH for let-7a and IHC staining for AR, CMYC and KRAS showed that let-7a expression was negatively correlated with CMYC and KRAS expression and that CMYC expression was positively correlated with KRAS expression. The results confirmed that let-7a is indeed a negative regulator of CMYC and KRAS *in vivo*.

In conclusion, the androgen-induced AR activating signal pathway directly upregulates let-7a miRNA expression. Let-7a targets CMYC and KRAS and plays an important role in the process of DHT inhibiting proliferation of ER^-, PR^-, AR^+ MDA-MB-453 and MDA-MB-231 breast cancer cells (Fig. 5). In FFPE breast cancer tissue, we further confirmed that let-7a is indeed a negative regulator of CMYC and KRAS *in vivo*. These findings contribute to the understanding of ER^-, PR^-, AR^+ breast cancer pathogenesis and will hopefully help in the design of new therapies for estrogen-insensitive neoplasms.

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