Androgen receptor-induced tumor suppressor, KLLN, inhibits breast cancer growth and transcriptionally activates p53/p73-mediated apoptosis in breast carcinomas

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Androgen receptor (AR) expression by immunohistochemistry correlates with better prognosis and survival among breast cancer patients. We and others have shown that AR inhibits proliferation and induces apoptosis in breast cancer cells. However, the mechanism of AR’s anti-tumor effect in breast cancer is still not fully understood. Our recent study indicates that AR upregulates expression of tumor suppressor gene PTEN by promoter activation in breast cancer. KLLN, encoding KLLN protein, is a newly identified gene, which shares a bidirectional promoter with PTEN and is transcribed in the opposite direction. So far, the function of KLLN has never been studied in tumorigenesis. Here, we define KLLN as a tumor suppressor in breast carcinomas, which inhibits tumor growth and invasiveness. After analyzing 188 normal breast and 1247 malignant breast cancer tissues, we observed the loss of KLLN in multiple breast cancer subtypes and this decreased KLLN expression associates with tumor progression and increasing histological grade in invasive carcinomas. We characterize KLLN, for the first time, as a transcription factor, directly promoting the expression of TP53 and TP73, with consequent elevated apoptosis and cell cycle arrest in breast cancer cells. We demonstrate, in vitro and in murine xenograph models, that both KLLN and PTEN are AR-target genes, mediating androgen-induced growth inhibition and apoptosis in breast cancer cells. Our observations suggest that KLLN might be used as a potential prognostic marker and novel therapy target for breast carcinomas.

INTRODUCTION

The ovarian production of androgens declines after menopause, at which time over two-thirds of female breast cancers are diagnosed, suggesting that the loss of androgens may play a role in breast cancer development. The androgen receptor (AR), a member of the nuclear receptor superfamily, regulates its target genes through specific androgen response elements (ARE) in promoter regions. AR has been widely studied as an oncogene in prostate cancer and has an established role in prostate carcinogenesis. However, burgeoning evidence suggests that AR may have an anti-tumorigenic effect in breast carcinomas. Maintaining functional AR in patients’ breast tumors has been reported to be significantly associated with increased survival and good prognosis (1–3). Earlier studies also suggest that androgens could induce apoptosis in breast cancer cells (4,5). Thus far, the mechanism of how androgens suppress proliferation and induces apoptosis in breast cancer is still not fully understood.

KLLN, encoding the KLLN protein, is a newly identified gene located in 10q23.31. KLLN is a high-affinity DNA-binding protein, which may regulate cell cycle in monkey kidney cells (6). We have previously shown that the KLLN promoter is hypermethylated in patients with the Cowden syndrome without germline mutations in PTEN, the first predisposition gene for this syndrome, which confers a high risk of breast, thyroid and other cancers (7). KLLN and PTEN share the same promoter region but are transcribed in opposite orientation.

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directions. Recently, we reported that AR stimulates PTEN expression by promoter activation, resulting in decreased AKT pathway activity and proliferation inhibition in breast carcinomas (8). Because KLLN’s function in endocrine-related carcinomas such as breast cancer has never been investigated, we now sought to address the hypothesis that KLLN is an AR-target gene, and AR-induced KLLN expression results in growth inhibition and apoptosis in breast carcinomas.

RESULTS

KLLN expression is downregulated in patient breast carcinomas and inhibits cancer cell growth

To begin to explore the role KLLN plays in human breast carcinomas, and to determine patient relevance, we first used publicly available databases to perform in silico comparisons of KLLN expression between normal breast and breast cancer tissues. A search through the Gene Expression Omnibus identified 156 normal breast and 1070 breast carcinoma tissue samples. Box-and-whisker plots showed that KLLN transcript expression is significantly decreased in breast carcinomas compared with normal breast tissues (Fig. 1A). Loss of KLLN expression is significantly associated with increased tumor histological grade ($P < 0.0001$, Fig. 1A). After analyzing KLLN expression in normal breast ($n = 68$), primary invasive ductal carcinomas (IDC) ($n = 822$) and metastatic carcinomas ($n = 393$), we found that the loss of KLLN is also correlated with breast cancer metastasis ($P < 0.001$, Fig. 1B). We then compared KLLN expression in matched normal and malignant tissues from 32 patients who were diagnosed with sporadic malignant breast carcinomas. We found that KLLN is significantly downregulated in tumors compared with matched normal breast tissues in almost all the patients (Fig. 1C), and we did not detect any KLLN exon mutations in these samples (data not shown). Next, we classified the tumors into different subgroups based on ER/PR/HER2/Ki67 molecular status. Downregulation of KLLN was observed in all groups of breast cancer compared with adjacent normal breast tissues, suggesting that loss of KLLN expression is common in malignant breast carcinomas and may not associate with any particular subtype (Supplementary Material, Fig. S1). In the normal mammary glands, both stroma and epithelium express KLLN protein (Fig. 1D, upper-left). In the earliest phase of ductal carcinoma in situ (DCIS), epithelial hyperplasia is associated with loss of KLLN expression in the inner small papilloma-like region, while the outer layer epithelial cells and stroma remain KLLN positive (Fig. 1D, upper-right). Late-stage DCIS (Fig. 1D, lower-left) and IDC show loss of KLLN expression in both stroma and epithelium (Supplementary Material, Table S1 and Fig. 1D, lower right). Our findings suggest that loss of KLLN in the epithelium is an early event in breast cancer development, and loss of KLLN expression in stroma is perhaps a later event during tumor progression.

In order to further elucidate the role that KLLN plays in breast carcinogenesis, we surveyed KLLN protein expression in 10 breast cancer cell lines. We found that MCF-7, MDA-MB-453, BT20 and SKBR3 cells express KLLN protein, while the rest of the breast cancer cell lines do not have detectable KLLN (Fig. 2A). Immunofluorescence staining and subcellular fractionation clearly show KLLN’s nuclear localization, suggesting its active role in the breast cancer cell nuclei (Fig. 2B and Supplementary Material, Fig. S2).

Next, we used a cell proliferation assay to determine KLLN’s effect on breast cancer cell growth. Overexpression of KLLN significantly inhibited cell growth in both MCF-7 (ER-positive luminal) and MDA-MB-453 (ER-negative molecular apocrine) cell lines, whereas knocking down KLLN by siRNA promoted cell proliferation (Fig. 2C). In vitro migration assay revealed that KLLN overexpression decreases breast cancer cells’ migration ability (Fig. 2D). After KLLN knockdown, tumor cells showed a dramatic increase in cell migration (Fig. 2D).

Finally, we investigated KLLN’s effect on breast cancer cells’ clonogenic ability. After ectopic-overexpression of KLLN, tumor cell clonogenic formation was decreased by 50% (Fig. 2E). Therefore, our data so far indicate that KLLN expression is downregulated in breast carcinomas, and the loss of KLLN may provide tumor cells with an advantage in proliferation and migration, with implications for progression and metastasis.

KLLN induces apoptosis by directly stimulating p53 and p73 expression

Our data above showed that KLLN inhibits breast cancer cell growth and metastasis. Previous study using monkey kidney COS-7 cell suggested that KLLN induces apoptosis and may also regulate cell cycle (6). To investigate the mechanism of KLLN’s anti-tumorigenic effect, we first examined its function on the cell cycle. Flow cytometry showed that overexpression of KLLN dramatically increases apoptosis in both MCF-7 and MDA-MB-453 cells (Fig. 3A). After KLLN transient transfection, we also observed cell accumulation in S-phase (Fig. 3A). To determine whether this increased S-phase is due to a promoted proliferation or S-phase arrest, we treated cells with nocodazole after KLLN expression. On the one hand, if KLLN promotes cell proliferation, the increased S-phase should be abolished by nocodazole after KLLN expression. On the other hand, if KLLN induces S-phase arrest, it should decrease nocodazole-induced cell G2 accumulation due to the S/G2 blockage. Nocodazole induces cell cycle arrest at G2 phase in MCF-7 (72%) and MDA-MB-453 (65%) cells (Fig. 3A). As we expected, KLLN-induced cell S-phase accumulation cannot be reversed by nocodazole-induced G2 arrest, indicating that KLLN arrests the cell cycle at S-phase (Fig. 3A). Western blots showed that, in both cell lines, overexpressed KLLN results in cyclin A (CCNA) accumulation and induces PARP cleavage, with consequent KLLN-induced apoptosis and cell cycle S-phase arrest in breast cancer cells (Fig. 3B). While KLLN is a known p53 target gene (6), we were surprised to find that p53 protein levels were significantly increased after KLLN expression in MCF-7 cells (Fig. 3B). MDA-MB-453 is a widely used p53-null breast cancer cell line (9,10). We noted that, without the functional p53 protein, KLLN still induces apoptosis in MDA-MB-453 cells, suggesting a p53-independent mechanism (Fig. 3A and B). Tumor suppressor p73 has long been known to induce apoptosis independent of p53 (11–13). We found that KLLN increases p73 protein...
KLLN, as a transcription factor, regulates the expression of its target genes, such as TP53 and TP73, potentially setting up a closed feedback loop. To test our hypothesis, we initially used qRT-PCR to determine whether KLLN stimulates TP53 and TP73 mRNA expression. After KLLN overexpression, endogenous TP53 and TP73 mRNA levels increased significantly, indicating that KLLN induces (directly or indirectly) TP53 and TP73 expression at the transcriptional level (Fig. 3C). This transcriptional stimulation is most likely mediated by promoter regulation, as KLLN overexpression increased the TP53 and TP73 promoter activities (Supplementary Material, Fig. S3). Finally, we used a ChIP assay to investigate whether KLLN directly binds to the TP53 and TP73 promoter in order to activate their transcription. Our ChIP assay clearly shows an interaction between KLLN and −218 to +5 region of the TP53 promoter (Fig. 3D, left panel). We also found an interaction between KLLN and the TP73 promoter, where KLLN has high binding affinity in the −750 to −659 region of this promoter (Fig. 3D, right panel). Taken together, we have successfully characterized KLLN as a transcription factor and have identified two KLLN targeting genes, TP53 and TP73, whose transcription are activated by KLLN and mediated KLLN-induced apoptosis in breast cancer cells.

Androgen-induced AR activation promotes both KLLN and PTEN expression in breast cancer cells

Our previous study showed that AR is positively associated with PTEN expression in breast carcinomas (8). In breast cancer cells, dihydrotestosterone (DHT) activates AR and promotes PTEN transcription, which is mediated by an ARE in the PTEN promoter (8). Since PTEN and KLLN share the same
promoter, we hypothesized that AR can induce KLLN expression in breast cancer. We test our hypothesis by using publicly available whole genome transcriptome data sets utilizing breast carcinomas derived from patients. A search through the Gene Expression Omnibus was performed for breast cancer samples and a total of 208 patient samples in four data sets were identified for meta-analysis. The forest plot showed that the transcripts of AR and KLLN in breast carcinomas are clearly positively correlated (correlation coefficient 0.166 with the upper limit 0.300 and the lower limit 0.026; Fig.4A). Based on these observations, we therefore surmised that AR could simultaneously regulate both PTEN and KLLN transcription by activating this bi-directional promoter in breast cancers. In order to investigate AR-regulated PTEN and KLLN transcription, we constructed two luciferase reporter constructs driven by the KLLN or PTEN promoter in opposite directions (Fig. 4B). We chose MCF-7, expressing wild-type AR, and MDA-MB-453 cells, which has a mutated but functional AR (Q865H) (14). DHT treatment stimulated the promoters’ transcriptional activity in both directions in MCF-7 (AR+) and MDA-MB-453 (mtAR) cells, while AR antagonist Casodex suppressed this promoter activity (Fig.4C). This DHT effect was not observed in AR-negative MDA-MB-468 cells, suggesting that AR promotes the transcription of both KLLN and PTEN as a transcriptional activator in breast cancer cells (Fig. 4C). We further confirmed that activation of AR by DHT increases both endogenous KLLN and PTEN mRNA expression by over 3-fold in breast cancer cells (Fig. 4D). In addition, androgen increased both KLLN and PTEN protein levels, and upregulated the expression of p53 and p73, indicating androgen-induced apoptosis in these two breast cancer lines (Fig. 4E). In contrast, Casodex inhibits PTEN and KLLN expression in both mRNA and protein level (Fig. 4D and Supplementary Material, Fig. S4A). We noted that the AR-induced p53 and p73 expression might be mediated by KLLN, as KLLN silencing abolished these androgenic effects (Supplementary Material, Fig. S4B).

Next, we injected MCF-7 (AR wt) or MDA-MB-231 (AR-) cells in female nude mice in order to generate xenograft
models to examine whether androgen induces KLLN and PTEN expression and tumor inhibition in vivo. DHT significantly suppressed tumor growth in this MCF-7 breast cancer xenograft during the 5-week treatment (Fig. 5A). This androgen-induced tumor suppression was not observed in AR-negative MDA-MB-231 xenografts (Fig. 5B). By using immunohistochemistry (IHC), we found that DHT not only inhibits tumor growth in AR xenografts, but also decreased tumor cell density in the remaining cancer tissues (Fig. 5C, first column). Female mice have very low levels of endogenous androgens, and so, the tumor cells expressed low levels of AR (Fig. 5C, second column). DHT induced AR expression as well as its nuclear localization, indicating the re-activation of the AR pathway (Fig. 5C, second column). This AR re-activation results in increased KLLN and PTEN expression and decreased Ki67 in tumors (Fig. 5C and D). Due to the absence of AR, DHT was not able to induce KLLN or PTEN expression in MDA-MB-231 (AR-) xenograft (Fig. 5E and F). Thus far, our observations indicate that AR can stimulate both KLLN and PTEN expression in AR-positive breast cancer cells, suggesting that AR’s anti-tumorigenic effect in MCF-7 cells is mediated through multiple pathways.
DISCUSSION

It is well known that sex steroid hormones play important roles in the development and progression of human breast carcinoma. Among the sex steroid hormones, estrogens have been intensively studied and its carcinogenic effects in breast carcinomas are well understood. A majority of breast carcinoma tissues express ER, whereas anti-estrogens treatments such as tamoxifen and aromatase inhibitors are effective endocrine therapies. AR is also expressed in a majority of patients’ breast cancer tissues (2,15,16), suggesting important roles of androgens in breast carcinomas. Clinical evidence suggests that AR expression by IHC in patients’ breast tumors is significantly correlated with good prognosis and better survival (1–3). However, the significance of androgens and AR in breast cancer development and progression remains largely debatable.

Androgens have been shown to inhibit ER-mediated tumor proliferation, induce apoptosis and cell cycle arrest in AR-breast cancer cell lines, such as CAMA-1, ZR75-1 and T47D (8,10,17–20). Two recent reports suggest that an androgenic effect on breast cancer cell growth may depend on tumor cell ER status, as DHT exerts anti-proliferative
effects in AR+/ER+ breast cancer cell lines while induces prolifera-
tion in AR+/ER− cells (21,22). However, Lehmann et al. ex-
aminied the effect of Casodex (AR antagonist) in 23 different
breast cancer cell lines and found that Casodex does not have
anti-proliferative effects in some AR+/ER− cells (23). Inter-
restingly, in the MCF-7 cell line, which is the most widely
used AR+/ER+ breast cancer cell model, different groups
also reported androgens’ anti-proliferative (13,19,24–26) or
proliferative effects (27,28). Although the reason for this is
unclear, it may involve the difference in the quality (R1881
versus DHT) and quantity (60-day capsule versus 90-day
capsule) of androgens that have been used in the studies.
These lines of controversial evidence most likely are due to
the complexity of the AR pathways and lack of acknowledg-
ment of broad mechanisms of androgenic effect in breast
cancer, in vivo and in vitro. First, aromatizable androgens
(DHEA and testosterone) and the unaromatizable androgen
DHT have been shown to have opposite effects on breast
cancer growth (13). In females, it is well known that aromat-
able androgens are precursors of estrogens and mainly act as
estrogens due to aromatization. To prevent this aromatiza-
tion-induced estrogenic effect, we have used unaromatizable
androgen, DHT, in our current study. Secondly, long-term ex-
posure to DHT inhibits the function of the hypothalamic-
pituitary-ovarian axis as a feedback and significantly decreases
luteinizing hormone and follicle stimulating hormone levels in
both male and female rats (29,30). As a result, long-term DHT
treatment in female breast cancer patients may suppress testos-
tered synthesis, which could lead to reduction of estradiol and
an anti-proliferative effect in vivo. Most importantly, very few
AR-regulated targets have been identified in breast cancer,
making it extremely challenging to demonstrate androgenic
effect, especially in different subtypes of breast carcinomas.
Therefore, this fuels the urgency to characterize androgens/
AR-target genes and to investigate the mechanisms of andro-
genic actions in breast carcinomas.

We previously reported that in breast cancer, androgen
activates AR and directly stimulates the expression of tumor
suppressor PTEN through an ARE in the promoter region,
resulting in cell growth inhibition (8). Cowden syndrome
patients who have mutations and variants in this ARE region
have a high frequency of prevalent breast cancer (8). In
the current study, we have found that AR can also activate the tran-
scription of a newly identified gene, KLLN, which shares a
bi-directional promoter with PTEN. For the first time, we are
able to show that KLLN acts as a tumor suppressor, which is
downregulated in broad subtypes of malignant breast carcino-
amas, especially in the late stages and in metastatic tumors.
KLLN overexpression in breast cancer cells suppresses cellular
proliferation and inhibits tumor cell migration, corroborating
our in silico analysis of patient breast carcinomas (Fig. 1A–D).

In vitro studies, to date, suggest that KLLN binds to DNA and
prevents DNA synthesis and replication (6). Germ line patho-
genic mutations in KLLN have been reported to be rare in
BRCA1-like breast cancer families (31). Here, we elucidate
the function of KLLN as a transcription factor, which directly acti-
vates the transcription of TP53 and TP73. We found that andro-
gen stimulates KLLN expression in breast cancer cells, which
induces S-phase arrest and apoptosis (Fig. 6). p53 is well
known for inducing cell cycle arrest at the G1/S transition

Figure 6. Schematic depicting the mechanism by which KLLN induces apop-
tosis and cell cycle arrest in breast cancer cells.

point. KLLN-induced S-phase arrest, however, appears not to be
mediated by KLLN’s simulative effect on TP53 expression.
First, KLLN induces S-phase arrest before the elevation of p53
expression (data not shown). Secondly, MDA-MB-453 cells,
which are p53-null, still showed S-phase arrest after KLLN
overexpression, indicating a p53-independent mechanism.
Therefore, KLLN-induced cell cycle arrest is an early response
to KLLN overexpression and appears to be independent of p53.
This KLLN-mediated anti-tumor effect is not dependent on ER
status and is observed in cell lines expressing wild-type or
mutant AR. In ER-positive MCF-7 cells, estradiol did not
affect KLLN expression or function (data not shown). Our
data, therefore, suggest that androgens play a sentinel role of
anti-tumorigenesis in breast neoplasia. This anti-tumorigenic
effect is mediated through a complex network, which involves
multiple pathways and regulates breast carcinogenesis at
several levels, including tumor cell proliferation, migration
and apoptosis. Among hundreds of AR-target genes, very few
have been studied in breast cancers, with the majority studied
in male-specific cancers, typically prostate cancer. Androgenic
effect on its targeting gene regulation appears to be tissue spe-
cific, providing additional challenge to study androgen/AR in
breast cancer. For example, AR suppresses PTEN transcription
in prostate but suppresses PTEN transcription in breast cancer
(8). MYC is a well-known AR-activated gene in prostate
cancer; however, its expression is suppressed by AR in breast
cancer (32). As suggested by our current data, future investiga-
tion elucidating the function of androgens/AR and its down-
stream pathways as well as the relationship to the complex
estrogen signaling networks, in breast carcinogenesis and
perhaps other women’s cancers may result in development of
new biomarkers of prognosis and response as well as
adjunctive-targeted therapies for breast carcinomas.

MATERIALS AND METHODS

In silico analysis of KLLN expression in breast carcinomas

A search through the Gene Expression Omnibus was performed
to identify public available whole genome transcriptome data
sets utilizing non-cultured breast carcinomas derived from
patients. Only these studies that contained more than 200
patients (with histology and pathology information) were
included. We specifically excluded cultured cancer cells and patients who received prior/on-going treatment. One hundred fifty-six normal breast tissues and 1215 breast carcinomas tissues (GSE1456, GSE3497, GSE4922 and GSE6532) were identified and reviewed for in silico analysis.

Human tissues and cell lines

Patient primary breast cancer and adjust normal tissues are from CHTN (Cooperative Human Tissue Network, Mentor, OH, USA). Among the patients with sporadic IDC, we chose 32 patients who provided both paired tumor and adjust normal tissues (median age, 60 ± 13.14 years). Based on patients’ pathology and histology reports, tumors were categorized into luminal A, luminal B, HER2 and triple-negative subtypes. IHC is detailed in the Supplemental experimental methods. Breast cancer cell lines MDA-MB-231, MDA-MB-453, MDA-MB-468, MCF-7, BT-474, HCC1395, BT20, MDA-MB-436, SKBR3 and AU565 cells were purchased from and authenticated by STR analysis by the ATCC (American Type Culture Collection, Manassas, VA, USA). All cell lines were resuscitated from low passage and all the experiments were performed with cells of passage number less than 25. Cells were monitored by microscopy and confirmed to maintain their original morphology. Cell molecular profiles including AR, ER and PTEN expression are tested by western blots and their original morphology. Cell molecular profiles including AR, ER and PTEN expression are tested by western blots and are consistent with previous literatures (10,23). The main cell models (MCF-7, MDA-MB-453 and MDA-MB231) are frequently tested for mycoplasma infection by PCR. Cells were cultured in ‘complete-medium’ per ATCC recommendation, with 10% FBS. DHT is from Steraloids (Newport, RI, USA). The KLLN coding sequence was PCR amplified from human genomic DNA (Promega, Madison, WI, USA), and subsequently cloned into p3xFLAG-CMV vector (Sigma-Aldrich).

Anti-KLLN siRNA is a pool of four siRNAs from Dharmacon (Lafayette, CO, USA). Control siRNA is a pool of four-scrambled non-specific siRNAs (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were transiently transfected using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY, USA) as described previously (33).

Western blotting

Western blotting was performed as described elsewhere (34). Mouse monoclonal anti-TUBULIN antibody is from Sigma-Aldrich (St Louis, MO, USA). Rabbit polyclonal anti- p73, CCNA and Lamin A antibodies are from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal anti-p53, PARP antibodies are from Santa Cruz Biotechnology. Rabbit polyclonal anti-KLLN antibody is from Abgent (San Diego, CA, USA). Mouse monoclonal anti-PTEN antibody is from Cascade Biosciences (Portland, OR, USA).

MTT assay

Cell growth rates were estimated by MTT assay. Cells were plated in 24-well plates and treated as indicated. Following treatment, each well was incubated with 25 ml of 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) for 1 h in a CO2 incubator at 37°C. The medium was aspirated and 0.5 ml DMSO was added per well. Proliferation rates were measured by colorimetric assay of formazan intensity in a plate reader at 560 nm.

In vitro trans-well migration assay

Migration assays were performed with the QCM Colorimetric Cell Migration Assay kit according to the manufacturer’s protocol (Millipore, Billerica, MA, USA). MCF-7 cells were transfected with control vector, KLLN-containing plasmid or anti-KLLN siRNA. After 48 h, 1000 cells were seeded in 24-well filter-inserts, and allowed to migrate toward 10% FBS for 8 h. Colorimetric measurements were taken according to the assay instructions.

Clonogenic assay

MCF-7 cells were transfected with control vector or KLLN-containing plasmid. After 24 h, cells were trypsinized and single-cell suspensions of 1000 cells were seeded into 100 mm culture dishes. After 12 days, colonies were fixed in 100% methanol for 15 min at room temperature and stained with 10% Giemsa (Gibco, Carlsbad, CA, USA). Dishes were washed with water and visible colonies containing ~50 or more cells were counted.

Flow cytometry

MCF-7 and MDA-MB-453 cells were transfected with control vector or KLLN plasmid. After 48 h, cells were treated with 40 ng/ml nocodazole for 12 h. Cells were trypsinized and fixed in 75% ethanol for overnight. After fixation, cells were treated with RNase A and stained by PI for flow cytometry to assess the cell cycle. Cell cycle and apoptosis were analyzed by ModFit LT (Verity Software House, Topsham, ME, USA).

Chromatin immune-precipitation (ChIP) assay

Chromatin immune-precipitation (ChIP) assays were performed with the EZ Chromatin Immunoprecipitation (EZ ChIP™) assay kit according to the manufacturer’s protocol (EMD Millipore). Rabbit polyclonal anti-KLLN antibody is from Abgent. Rabbit polyclonal anti-polymerase II and normal rabbit IgG are from EMD Millipore. The ChIP-eluted DNA was amplified by PCR using primers specific for the TP53 or TP73 promoter. PCR amplification was performed using the PCR Master Mix (Promega). Primers utilized for ChIP are contained in the Supplementary materials.

Meta-analysis of the correlation coefficient between AR and KLLN

All analyses were performed in R version 2.10.0 (35). A search through the Gene Expression Omnibus was performed for the words ‘breast cancer, patient samples’. Only those studies that contained more than 20 patients were included. We specifically excluded cultured cancer cells and the patients who received prior/on-going treatment. Results were limited to the Affymetrix HGU133Plus2.0 platform (GPL96/97), which contains probes for AR and KLLN genes. A total of
four breast cancer data sets were identified and reviewed for meta-analysis. Meta-analysis of the correlation coefficients was performed using the library meta, and the R script was described previously (8). Forest plots showed a summary estimate of the correlation between the expression of AR and KLLN in breast cancer tumor tissues.

Promoter activity assay

The PTEN and KLLN promoters were PCR amplified from normal genomic DNA and subsequently cloned into pGL3.1-Basic vector (Promega). Promoter activity was determined by dual luciferase assay using a luciferase enzyme assay system (Promega).

Mouse xenograft assay

MCF-7 or MDA-MB-231 cells were injected (5 × 10⁶ cells in 50 μl Matrigel and 50 μl medium mix, BD bioscience, San Jose, CA, USA) subcutaneously into both flanks of 4- to 6-week-old female NOD/SCID mice (Jackson Laboratory, Bar Harbor, ME, USA). The take rates for MCF-7 and MDA-MB-231 were 90.4 and 88.0%. Tumors were measured twice a week and the volume was calculated as 0.5 × (LxW²) mm³. Depending on the cell lines, the average of tumors size reached 50 mm³ after 2–3 weeks, and mice carrying xenographs were randomly separated into control and DHT groups based on the body weight. Mice in the DHT group were implanted with DHT pellets (10 mg/90 days; Innovative Research of America, Sarasota, FL, USA). After 5 weeks of treatment, mice were sacrificed. Tumors are weighted, fixed and dissected for IHC. The Cleveland Clinic Animal Care and Use Committee approve the protocol for animal experiments conducted at Cleveland Clinic.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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