MicroRNA145 Targets BNIP3 and Suppresses Prostate Cancer Progression

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Abstract

The putative tumor suppressor miR145 is transcriptionally regulated by TP53 and is downregulated in many tumors; however, its role in prostate cancer is unknown. On the other hand, BCL2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) is overexpressed in various tumors, including prostate cancer, and may transcriptionally repress the apoptosis-inducing factor (AIF) gene. Although BNIP3 transcription is controlled by hypoxia-inducible factor 1α (also elevated in prostate cancer), we postulated the posttranscriptional regulation of BNIP3 by miR145 through bioinformatics analysis, and herein we experimentally showed that miR145 negatively regulated BNIP3 by targeting its 3′-untranslated region. Artificial overexpression of miR145 by using adenoviral vectors in prostate cancer PC-3 and DU145 cells significantly downregulated BNIP3, together with the upregulation of AIF, reduced cell growth, and increased cell death. Artificial overexpression of wild-type TP53 in PC-3 cells (which lack TP53 protein) and DU145 cells (in which mutated nonfunctioning TP53 is expressed) significantly upregulated miR145 expression with consequent effects on BNIP3 and cell behavior as with miR145 overexpression. Analysis of prostate cancer (n = 134) and benign prostate (n = 83) tissue sample showed significantly decreased miR145 and increased BNIP3 expression in prostate cancer (P < 0.001), particularly in those with tumor progression, and both molecular changes were associated with unfavorable outcome. Abnormalities of the miR145-BNIP3 pair as part of TP53-miR145-BNIP3-AIF network may play a major role in prostate cancer pathogenesis and progression. Cancer Res; 70(7); 2728–38. ©2010 AACR.

Introduction

Posttranscriptional regulation of mRNA by microRNA (miRNA) is an important mechanism controlling gene function. Abnormalities in these small RNAs ∼22 nt in length has been implicated in the pathogenesis of a variety of diseases, notably neoplasms (1–3). Overexpression of oncogenic miRNAs (oncomirs) or underexpression of tumor suppressor miRNAs plays pivotal roles in tumorigenesis. One major tumor suppressor miRNA, miR145, is downregulated in such neoplasms as colorectal, mammary, ovarian, and B-cell tumors (4–8). Although microarray screening indicated that miR145 is also among the downregulated miRNAs in prostate cancer (9, 10), its role in prostate cancer is unknown.

On the other hand, BCL2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3), a BH3-only Bcl-2 family protein (11), is overexpressed in many cancers, including prostate cancer (12). Although conventionally classified as a pro–cell death protein, BNIP3 has recently been found to function as a transcriptional corepressor of the apoptosis inducing factor gene AIF (13). Transcription of BNIP3 is regulated by hypoxia-inducible factor 1α (HIF-1α; refs. 14, 15); however, our preliminary data indicate significant contribution of HIF-1α–independent mechanisms, and bioinformatics analysis shows that miR145 may be a regulator of BNIP3. Herein, we show that BNIP3 mRNA is a target regulated by miR145. Loss of miR145, a major cause of which may be dysfunction of its transcription activator TP53, results in the overexpression of BNIP3 in prostate cancer and may lead to the downregulation of the proapoptotic gene AIF. Aberrancy of this pathway is significantly associated with prostate cancer progression and a worse prognosis.

Materials and Methods

Cell lines and general reagents. Human prostate cancer cells LNCaP, DU145, and PC-3 were from the American Type Culture Collection and were maintained in RPMI 1640 with 10% FCS (Life Technologies). The adenovirus-immortalized human embryonic kidney epithelial cell HEK-293 was maintained in DMEM with 10% FCS. The phosphatidylinositol-3 kinase (PI3K) inhibitor LY294002 was from Sigma. Tris base, Tween 20, DTT, and EDTA were from Amresco. Phenylmethylsulfonyl fluoride, leupeptin, pepstatin, and aprotinin were from Roche Diagnostics.
miR145 Targets BNIP3

Tissue samples and clinical data. Two hundred seventeen archived formalin-fixed, paraffin-embedded samples, including 134 prostate adenocarcinoma (121 needle biopsies, 13 transurethral resection of prostate samples) and 83 benign prostate (transurethral resection of prostate) tissues, were used, as were 7 snap-frozen fresh tissue samples (2 cancerous, 3 benign prostate hyperplasia, and 2 normal) obtained from prostatectomy specimens. All tissue samples were from West China Hospital and were collected and used according to the ethical guidelines and procedures approved by the institutional supervisory committee. The Gleason scores of the prostate adenocarcinomas were as follows: 5 to 6 (8 cases, 6%), 7 (38 cases, 28%), and 8 to 10 (88 cases, 66%). The tumor-node-metastasis stages were as follows: stage II has 10 cases (7%), stage III has 79 cases (59%), and stage IV has 45 cases (34%). This cohort of patients ranged in age from 54 to 87 y (mean, 71.6 y) and were treated by combined androgen blockade (surgical castration plus flutamide). Patients were followed by clinical and laboratory monitoring on a regular basis starting at definitive diagnosis. Disease-specific survival time is defined as the time from definitive diagnosis to disease-specific death, and progression-free survival time is defined as the time from definitive diagnosis to any of the following events after initial treatment: prostate-specific antigen elevation, death, and progression-free survival time.

Stem-loop reverse transcription, conventional reverse transcription-PCR, and genomic DNA PCR. Total RNA was extracted by using the Trizol reagent (Invitrogen). The stem-loop reverse transcription-PCR (RT-PCR) technique was used to examine mature miR145 (16). The stem-loop RT primer was designed as 5′-GTCGATCCAGTTGAGGT-CCAGGATTACACTGAGAAGGAT-3′. RT was carried out in 20 μL volume containing 2.5 μg of total RNA, 1.6 mmol/L miR145 stem-loop primer, 2 μL 10 mmol/L deoxyribonucleotide triphosphate, 1 μL 0.1 mol/L DTT, and 1 μL M-Mulv reverse transcriptase (Fermentas) at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. The PCR primers for mature miR145 were designed as follows: sense, 5′-CGGCTCCAGTCTTTCAGG-3′; 5′-GTGACCGATCGCTCCAGGT-3′ (product length, 62 bp).

The random RT primer 5′-d(Invitrogen) was used for other genes, the PCR primers of which were designed according to their respective cDNA sequences (Genbank) as follows: HIF-1α (5′-CTATGACCTGGTGTGCT-3′, 5′-CTGGCCTATATCCCACTAT-3′; product length, 157 bp), BNP3 coding sequence fragment (5′-ACCAACAGCCTTCTGAAC-3′, 5′-GAGGTTCCGGTGC-3′, 202 bp), BNP3 fragment spanning CDS and 3′-untranslated region (UTR; 5′-ACCAACAGCCTTCTGAAC-3′, 5′-CTCGAGCCCAGAGTTCAACA-GCTCTCAG-3′, 444 bp), AIF (5′-CTGAAAGCCGGCAGGAAGTTAG-3′, 5′-TCCAGACATTTCCATGAC-3′, 253 bp), TP53 (5′-GTGGTGTGGTGGCTTATGAGC-3′ and 5′-ACAGGCACAAACGGAGACCT-3′, 188 bp), GADD45A (5′-GAGGACGAGGGGAGAAGGAA-3′, 145 bp), and β-actin (5′-CTGGCCTACGCCTTCTACAG-3′, 248 bp). Standard PCR protocols were used; products were resolved by 2% agarose gel or 10% PAGE and were visualized by staining with ethidium bromide or the fluorescent dye Goldview (SBS). Images were captured by scanning with Typhoon 8600 Multi-Imager (Molecular Dynamics) under fluorescence mode or with Bio-Rad Gel Doc XR (Bio-Rad). Semiquantitative analysis was performed with the ImageQuant 5.2 software (Molecular Dynamics).

The genomic sequence corresponding to pri-miR145 was amplified from genomic DNA prepared with an extraction kit (Promega) and primers as described (17). The β-globin gene (5′-GATCTGCTCTCCTGATGCTG-3′, 5′-ATCAACGCTCCATAGACTAC-3′, 196 bp) was used as internal control. The BNIP3 3′-UTR was amplified and sequenced by using the following primers: 5′-TCCACACCACCTTGTGGA-3′ and 5′-TCCAGACACCTGACATT-3′ (513 bp), and 5′-GCTACCTTAAGGAGTTTTGTCC-3′ and 5′-CCTCTAGAAAAATTATTTTTTCACC-3′ (435 bp).

Real-time quantitative PCR. Real-time quantitative PCR (Q-PCR) was used together with stem-loop RT to quantitate mature miR145. Q-PCR was performed on Light Cycler 2.0 (Roche), and data were analyzed with the Light Cycler software 4.05 (Roche) as described (18). The β-actin gene was used as control. Copy number of target genes (relative to β-actin) was determined by the 2-△△Ct method, with △Ct = △Ctexp − △Ctcon = (Ctexp-target − Ctexp-control) − (Ctcontrol-target − Ctcontrol-control), in which “exp” represents the experimental group, “con” the control group, and “target” the gene of interest.

Locked nucleic acid in situ hybridization. Tissue sections were prepared from paraffin-embedded tissue blocks, deparaffinized, transferred into diethyl pyrocarbonate-treated water, treated with proteinase K (20 μg/mL; Roche) at 37°C for 30 min then with 0.2% glycine for 1 min, and fixed with 4% paraformaldehyde. The sections were incubated in hybridization buffer (50% formamide, 5× SSC, 0.1% TWEEN, 9.2 mmol/L citric acid for adjustment to pH 6, 50 μg/mL heparin, 500 μg/mL yeast RNA) at 37°C for 2 h. Digoxigenin-labeled, LNA-modified miR145 probe (20 nmol/L; 5′-AGGGATCTCCGGAAACTGAC-3′, Exiqon) was added and incubated at 53°C for 18 h. Sections were washed with 2× SSC once then with 2× SSC with 50% formamide at 53°C thrice (30 min each). The anti-DIG-AP antibody (1:1,000, Roche) was added after PBST (PBS with 0.1% Tween 20) wash and incubated at 37°C for 1 h and then at 4°C overnight. Sections were washed five times with PBST, and nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indonyl phosphate was used for hybridization signal detection. Two percent methyl green (Sigma) was used for nuclear counterstain. The LNA probe for U6 (5′-CAGCAGATTTGCGGTTCAC-3′) was used as control (hybridization temperature at 50°C).

The locked nucleic acid in situ hybridization (LNA-ISH) signal intensity was recorded semiquantitatively (19), with 0 indicating no signal and 1 to 3 for weak, moderate, and strong signals, respectively. The extent of LNA-ISH signal was defined as the percentage of cells showing signal and recorded as 0 (0%), 1 (1–30%), and 2 (>30%). An integrated score of LNA-ISH signal (obtained by the product of the intensity score and the extent score) of 4 or more was designated as miR145 positive.
Western blot. The primary antibodies used were as follows: BNIP3 (mouse monoclonal, 1:3,000, Sigma), HIF-1α (mouse monoclonal, 1:1,500, Chemicon, Inc.), phosphorylated AKT (rabbit polyclonal, 1:600, Cell Signaling Technology, Inc.), AKT1/2 and pAKT (goat polyclonal, 1:800, Santa Cruz Biotechnology), TP53 (mouse monoclonal, 1:1,000, Boster), AIF (rabbit monoclonal, 1:500, Epitomics, Inc.), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; mouse monoclonal, 1:10,000, Kangcheng), and β-tubulin (mouse monoclonal, 1:1,000, Huatesheng). Horseradish peroxidase–labeled secondary antibodies were from Zymed Laboratories, Inc. Western blotting was carried out as previously described (18).

Immunohistochemistry and immunocytochemistry. The antibodies and dilutions used for immunohistochemistry were BNIP3 (mouse monoclonal, 1:300, Sigma), HIF-1α (mouse monoclonal, 1:200, Chemicon), and cleaved caspase-3 antibody (rabbit polyclonal, 1:200, Cell Signaling Technology). Immunostaining was carried out as previously described (18).

Recombinant adenoviral vectors for overexpression of miR145 and TP53. The pri-miR145 sequence −179 to +287 (+1 being the first base of the mature miR145) was amplified from HEK-293 cell genomic DNA with the primers described (17). PCR product was cloned into pMD18-T (TaKaRa), verified by sequencing, and subcloned into shuttle plasmid pAdTrack-CMV (designated as pAdTrack-miR145; ref. 20). pAdTrack-miR145 linearized with PmeI was used to transform BJ5183-AD-1 cells harboring the adenoviral pAdeasy-1 vector (Stratagene) for homologous recombination. Colonies were screened by plasmid miniprep and PacI restriction analysis to obtain clones with recombinant miR145 (designated as pAdeasy-miR145). PacI linearized pAdeasy-miR145 was used to transfect HEK-293 cells to obtain packaged recombinant miR145 adenovirus (designated as AD-miR145). AD-miR145 was amplified by repeated infection and verified by PCR. The pAdeasy-CMV empty vector was used as control (designated as AD-control). The titers and the multiplicity of infection were determined according to the manufacturer’s protocols.

The adenovirus vector for wild-type TP53 was constructed in a similar fashion. The coding sequence of wild-type TP53 was cloned from 293 cells by RT-PCR with the following primers: 5′-AAGCTTATGGAGGAGCCGCAGTC-5′ and 5′-TCTAGACAGTGGGGAACAAGAAGTG-3′.

Cell viability assay. Cells were collected and stained with trypan blue (Sigma, 200 mg/mL). The number of viable cells was determined by microscopic examination.

Flow cytometry. Collected cells were incubated with Annexin V-PE, 7-AAD, or both (BD Pharmingen) in 1 × Annexin V binding buffer (BD Pharmingen) for 30 min at 4°C in the dark and then analyzed on BD FACSAria flow cytometer (BD Pharmingen). Unstained and nontreated cells were used as control. Data were collected and analyzed with the manufacturer’s software, and Annexin V–PE(+)/7-AAD(−) cells were gated as the apoptotic cell population.

Figure 1. miR145 and BNIP3 expression in normal prostate (NP), benign prostate hyperplasia (BPH), prostate cancer tissue (PCa), and prostate cancer cell lines. A, stem-loop RT-PCR analysis (with actin as control) showing differential expression of mature miR145 in benign prostate tissue (NP and BPH) versus prostate cancer, and prostate cancer cells DU145, PC-3, and LNCaP; genomic DNA PCR analysis (with globin as control) of miR145 gene (genomic) showed no difference. B, further validation of loss of miR145 in prostate cancer by LNA-ISH (nuclear counterstain with methyl green) with U6 as control. The miR145 and U6 signals were in purple blue. C, in contrast to miR145, BNIP3 mRNA (top, RT-PCR, same actin control as for A) and protein (bottom, Western blotting, GAPDH as control) were significantly higher in prostate cancer tissue and cells than in benign prostate tissue. D, overexpression of BNIP3 protein in prostate cancer, represented by cytoplasmic and nuclear (inset) brown staining, compared with benign prostate tissue, was further shown by immunohistochemistry (nuclear counterstain with hematoxylin).
Terminal deoxynucleotidyltransferase–mediated biotinylated dUTP nick end-labeling. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) was performed by using in situ cell death detection kit (Roche) as previously described (18).

Luciferase reporter constructs and site-directed mutagenesis. The two seed sequences (6–12 and 796–802 nt) of BNIP3 3′-UTR with flanking sequences were amplified from genomic DNA of PC-3 cells. Site A (~27 bp to +84 bp, with +1 being the first base after stop codon) was prepared with the primers BNIP3-Xba-I-P1 (5′-TCTAGACTGACAATCTCCACACGAC-3′) and BNIP3-Xba-I-P2 (5′-CTCGAGCGAGATCTACAGCCTTCTC-3′), whereas site B (+658 bp to +911 bp) was prepared with BNIP3-Xho-I-P3 (5′-CTCGAGCTCTGCTGAAGGCACCTACTC-3′) and BNIP3-Xho-I-P4 (5′-TCTAGAGTTCACGTTCCTTGTTTC-3′). PCR products were cloned into pMD18-T then subcloned into pGL3-Promoter (Promega) and designated as pGL3-site A and pGL3-site B, respectively, in which the site A and site B sequences were inserted as the 3′-UTR downstream of the luciferase coding sequence, respectively. A construct with site A and B sequences in tandem (pGL3-site A+B) was prepared by cloning of the ligated site A and B sequences.

Overlapping PCR was used for site-directed mutagenesis of the seed sequence (from AACTGGA to AGATCTC) in site A (designated as pGL3-Mut A) and site B (designated as pGL3-Mut B). A construct combining the two was prepared and designated as pGL3-Mut A+B. The PCR primers used were as follows: BNIP3-site A-Mut1 (5′-GAAGATCTCTTCATCAAAAGGTGCTG-3′), BNIP3-site A-Mut2 (5′-GAAGATCTGCTGACTTGGTTCGTTAG-3′), BNIP3-site B-Mut1 (5′-CTACTTTAAAAAGATCTCAATGGAAAAA-3′), and BNIP3-site B-Mut2 (5′-CATGGAGATCTTTTAAAGTAGACAC-3′).

Dual reporter gene assay. PC-3 cells were cultured in 24-well plates and transfected with 0.4 μg of the reporter constructs by using Lipofectamine 2000 (Invitrogen). The pRL-CMV plasmid (Promega) containing the Renilla luciferase gene (0.02 μg) was cotransfected as internal control. Cells were infected with AD-miR145 and AD-control (multiplicity of infection, 100) 4 h after transfection, collected 24 h later, and the firefly and Renilla luciferase activities were assayed on Luminometer TD-20/20 (Turner Design).

Statistical analysis. The SPSS 10 program was used for general statistical and survival analysis. Fisher’s exact test was used for between-group comparisons, and Spearman rank order correlation was used for correlation analysis. The Kaplan-Meier method with log-rank test was used for univariate survival analysis, and the Cox proportional regression model was used for multivariate survival analysis.

Results

miR145 was significantly downregulated in prostate cancer. Expression of mature miR145 was investigated by using stem loop RT-PCR, which showed high expression levels of miR145 in benign prostate hyperplasia and normal prostate tissue but very low levels in prostate cancer tissue (Fig. 1A). In PC-3, DU145, and LnCaP cells, no miR145 could be detected (Fig. 1A).

Real-time PCR further showed that miR145 in prostate cancer tissue was only 1/53.8 of that in benign prostate tissue and was barely detectable in the three prostate cancer cell lines (<1/10,000 of that in benign prostate tissue; data not shown).

In cancer tissue samples, the presence of stromal cells (which may express miR145) could interfere with the evaluation of expression level with the stem loop RT-PCR technique. To further examine the expression status in tissue samples, the LNA-ISH technique was used, which showed that miR145 was expressed at high levels in benign prostate epithelia but at significantly lower levels or was absent in the majority of prostate cancer parenchyma (Fig. 1B). The miR145 positivity rate in benign prostate tissue samples (56 of 83, 67.5%) was significantly higher than in prostate cancer tissue samples (33 of 106, 31.1%, P = 0.000; Table 1).

Sequence analysis of the miR145 gene showed no mutation or deletion (data not shown) in the three prostate cancer cell lines.

miR145 expression was inversely related to BNIP3 overexpression in prostate cancer. BNIP3 overexpression in prostate cancer tissue samples and cell lines was shown by RT-PCR, Western blot analysis, and immunostaining (Fig. 1C and D). The proportion of prostate adenocarcinoma cases (89 of 134, 66.4%) with moderate to strong BNIP3 immunostaining (score 2 and 3) was significantly higher than that of benign prostate tissue (16 of 83, 19.3%, P = 0.000).

Table 1. Association of miR145 and BNIP3 expression levels with prostate cancer progression

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<th>miR145(+)</th>
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<tr>
<td>BPH</td>
<td>56/83 (67.5%)</td>
<td>16/83 (19.3%)</td>
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<td>PCa total</td>
<td>33/106 (31.1%)</td>
<td>89/134 (66.4%)</td>
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<td>PCa with progression</td>
<td>7/50 (14.0%)</td>
<td>49/64 (76.6%)</td>
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<td>PCa without progression</td>
<td>26/56 (46.4%)</td>
<td>40/70 (57.1%)</td>
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<td>BPH vs PCa total</td>
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<td>BPH vs PCa with progression</td>
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<td>PCa vs with vs without progression</td>
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NOTE: miR145(+) was defined as having a LNA-ISH staining score of 4 or above. BNIP3(+) was defined as moderate to strong immunostaining (score 2 and 3). P values were determined by Fisher’s exact test.

Abbreviations: BPH, benign prostate hyperplasia; PCa, prostate cancer.

*Number of miR145(+) or BNIP3(+) cases/total number of cases (positive rate %).
Importantly, correlation analysis of BNIP3 protein level (determined by immunohistochemistry) and miR145 level (determined by LNA-ISH) showed inverse relationship between the two ($r_s = -0.314$, $P < 0.01$; Table 1; Fig. 1).

Identification of potential BNIP3 3′-UTR seed sequences. The 824 nt 3′-UTR of the BNIP3 mRNA (full-length 1,535 nt, coding sequence 127–711 nt) was analyzed by using TargetScan 5.1 (http://www.targetscan.org/), which identified miR145 as the major potential regulatory miRNA of BNIP3. The 6 to 12 nt and 796 to 802 nt of the BNIP3 3′-UTR were identified as two potential seed sequences for miR145, designated as sites A and B, respectively (A), which were conserved across species (B). C, dual reporter gene assays were performed with pGL3 expression constructs with BNIP3 3′-UTR regions containing the seed sequences inserted downstream of the luciferase coding sequence, and the activity of the basic pGL3 construct as baseline (pGL3-Promoter). With the artificial expression of miR145 (by coinfection with Ad-miR145), the reporter gene activity, represented by relative luciferase activity (firefly/Renilla), was significantly decreased when either site A or site B, or site A + B (in tandem) was present in the constructs, whereas mutations of the seed sequences (Mut A, Mut B, and Mut A + B) significantly restored reporter gene activity. Expression of miR145 alone had no effect on reporter gene activity when no seed sequences were inserted.

Dual reporter gene assays showed interaction of miR145 with 3′-UTR of BNIP3. To show posttranscriptional

Figure 2. Identification of miR145 seed sequences in BNIP3 3′-UTR and dual reporter gene assays for miR145-BNIP3 3′-UTR interaction. A and B, the 6 to 12 nt and 796 to 802 nt of the BNIP3 3′-UTR were identified as two potential seed sequences for miR145, designated as sites A and B, respectively (A), which were conserved across species (B). C, dual reporter gene assays were performed with pGL3 expression constructs with BNIP3 3′-UTR regions containing the seed sequences inserted downstream of the luciferase coding sequence, and the activity of the basic pGL3 construct as baseline (pGL3-Promoter). With the artificial expression of miR145 (by coinfection with Ad-miR145), the reporter gene activity, represented by relative luciferase activity (firefly/Renilla), was significantly decreased when either site A or site B, or site A + B (in tandem) was present in the constructs, whereas mutations of the seed sequences (Mut A, Mut B, and Mut A + B) significantly restored reporter gene activity. Expression of miR145 alone had no effect on reporter gene activity when no seed sequences were inserted.

Figure 3. Effects on BNIP3, AIF, and cell behavior by artificial overexpression of miR145 in PC-3 and DU145 cells. A and B, the efficiency of Ad-miR145 and Ad-control infection was shown by homogenous green fluorescence protein expression of the infected cells (A, top). Artificial overexpression of miR145 by Ad-miR145 (A) resulted in the significant downregulation of BNIP3 protein level compared with Ad-control (B, top, Western blot with semiquantitative histograms; bottom, immunocytochemistry with brown staining representing BNIP3 protein) but no change in the BNIP3 mRNA level (A, bottom, left, RT-PCR; right, Q-PCR) or HIF-1α mRNA (A) or protein (B) levels. AIF was simultaneously upregulated upon miR145 overexpression (A) and BNIP3 downregulation (B). C, cell growth was significantly inhibited concomitant with the miR145-BNIP3-AIF expression change. D, increased cell death upon the miR145-BNIP3-AIF expression change as shown by flow cytometry analysis of percentage of Annexin V-PE–stained apoptotic cells (top) and TUNEL assays (middle). The cell death was independent of caspase-3 activation, as shown by lack of immunostaining of cleaved (activated) caspase-3 (bottom; inset, positive control of activated caspase-3 immunostaining in PC-3 cells irradiated with UV).
regulation of BNIP3 mRNA by miR145, luciferase reporter gene constructs were prepared in which the potential seed sequences (sites A and B) of BNIP3 3′-UTR were cloned into luciferase reporter constructs, together with constructs in which the seed sequences were mutated.

With artificial coexpression of miR145 by infection with Ad-miR145, dual reporter assays showed significant downregulation of luciferase reporter gene activity by 63.6%, 50.7%, and 67.5% in the pGL3-site A, pGL3-site B, and pGL3-site A+B constructs, respectively (Fig. 2C), whereas reporter constructs lacking BNIP3 3′-UTR sequences were not affected. Moreover, mutation of the seed sequences significantly restored the luciferase gene activity in the constructs bearing the mutated 3′-UTR sequences (Fig. 2C).

Overexpression of miR145 by adenoviral vectors led to downregulation of BNIP3 protein, upregulation of AIF, and increased cell death. The biological effects of BNIP3 regulation by miR145 were further shown by assaying molecular and cellular changes in PC-3 and DU145 cells with artificial miR145 overexpression (Fig. 3A–D). Concomitant with the overexpression of mature miR145 by Ad-miR145 infection (Fig. 3A), BNIP3 protein level was significantly downregulated (Fig. 3B). In contrast, the BNIP3 mRNA, as well as the HIF-1α mRNA and protein, showed little change with miR145 overexpression (Fig. 3A and B).

Significantly, the AIF level was upregulated simultaneously (Fig. 3A) with miR145 overexpression. Synchronous with the BNIP3-AIF change upon artificial miR145 overexpression, PC-3 and DU145 cells showed reduced cell growth (Fig. 3C) and increased cell death (as assayed by flow cytometry and TUNEL; Fig. 3D), which was independent of caspase-3 activation (Fig. 3D).

Artificial overexpression of wild-type TP53 in PC-3 and DU145 cells restored miR145 expression, together with downregulation of BNIP3 and upregulation of AIF. The tumor suppressor TP53 has been identified as a transcriptional regulator of miR145 (21). Loss of TP53 may be an important mechanism underlying miR145 downregulation because PC-3 cells lack TP53 protein and the TP53 in DU145 cells is mutated and nonfunctional (Fig. 4A; refs. 22, 23). Indeed, treatment of PC-3 and DU145 cells with PI3K/AKT inhibitor LY294002 could not restore TP53 function and miR145 expression despite significant downregulation of phosphorylated AKT (p-AKT; Fig. 4A).

To further show that the loss of TP53 function may have led to the loss of miR145 expression, we used adenoviral vectors to artificially overexpress wild-type TP53 (Fig. 4B and C). As shown in Fig. 4C, this resulted in significant upregulation of miR145 and simultaneous downregulation of BNIP3 protein (Fig. 4C). Importantly, the AIF gene expression was also upregulated upon TP53 overexpression (Fig. 4C).

Figure 4D schematically summarizes the data presented in the above sections showing BNIP3 regulation by miR145, which may result from loss of TP53 function in prostate cancer cells.

Biological significance of miR145 and BNIP3 in prostate cancer. Clinical analysis of our data showed that miR145 and BNIP3 abnormalities were associated with prostate cancer pathogenesis and progression. In addition to the significantly different expression pattern of miR145 and BNIP3 in benign and cancerous prostate tissue samples (Table 1; Fig. 1), the positive rate of miR145 in prostate cancer with progression (7 of 50, 14.0%) was significantly lower than prostate cancer without progression (26 of 56, 46.4%, P = 0.000; Table 1), whereas the positive rate of BNIP3 in prostate cancer with progression (49 of 64, 76.6%) was significantly higher than prostate cancer without progression (40 of 70, 57.1%, P = 0.027; Table 1).

Kaplan-Meier analysis showed that decreased miR145 level and increased BNIP3 level, among other clinico-pathologic factors, were significant negative prognostic factors for both disease-specific and progression-free survival in prostate cancer patients (P < 0.05; Fig. 5). Cox proportion regression model incorporating classic clinico-pathologic parameters (Gleason score, prostate-specific antigen level, and tumor stage) showed that the expression of miR145 was also an independent favorable prognostic factor for progression-free survival (relative risk, 0.404; 95% confidence interval, 0.174–0.941; P = 0.036).

Discussion

miR145 regulation of BNIP3. The present study is the first to identify BNIP3 as a target of posttranscriptional regulation by miR145. Although BNIP3 transcription is regulated by HIF-1α (14, 15), posttranscriptional control by miR145 contributes significantly to BNIP3 regulation (Fig. 4D).

miR145 is downregulated in various human tumor types, including cancers of the gastrointestinal tract, liver, nasopharynx, lungs, urinary bladder, ovaries, uterine cervix, B cells, and soft tissue (5–8, 19, 24–30). In some tumors, downregulation of miR145 is correlated with tumor size, stage, proliferative activity, or poorer prognosis (6, 8, 30, 31). Gradual decrease of miR145 is observed in mammary neoplasia (19), whereas artificial overexpression of miR145 inhibits cell growth and tumor formation (8, 32). Downregulation of miR145 in various cancers has made it one of the most noticeable tumor suppressor miRNAs (1).

Identification of miRNA targets is one of the most important aspects in understanding the mechanisms by which miRNAs control cell behavior (2, 3, 33). Several genes, including IRS-1, OCT4, SOX2, nuclear Kruppel-like factor 4, C-MYC, and RTKN (rhotekin), have been identified as miR145 target genes (21, 32, 34, 35). Inhibition of the pro-proliferation and antiapoptosis factor IRS-1 by miR145 suppresses colon cancer cell growth (32). RTKN, a Rho-GTP–interacting and GTPase-inhibiting protein, is inhibited by miR145, the loss of which may promote breast cancer cell growth (35). Inhibition of stem cell factors OCT4, SOX2, and Kruppel-like factor 4 by miR145 represses pluripotency in human embryonic stem cells and promotes cell differentiation (34). However, the roles of miR145 in tumorigenesis of many neoplasms are still to be elucidated.

Our study identified BNIP3 as a novel posttranscriptional target of miR145 and miR145-BNIP3 as an important pair deranged in prostate cancer. Because BNIP3 transcription...
Figure 4. Artificial overexpression of wild-type TP53 in PC-3 and DU145 cells restored miR145 expression. A, despite significant downregulation of phosphorylated AKT (p-AKT), treatment with PI3K/AKT inhibitor LY294002 could not restore TP53 function and miR145 expression (bottom, RT-PCR) in either PC-3 cells (which lack the TP53 protein) or DU145 cells (in which mutated nonfunctional TP53 protein is expressed; top, Western blot with tubulin as control). B, artificial overexpression of wild-type TP53 by Ad-TP53 (compared with Ad-control) resulted in significant inhibition of cell growth and increased cell death. C, miR145 expression was significantly restored upon wild-type TP53 expression (top, left, RT-PCR; right, Q-PCR), with simultaneous downregulation of BNIP3 protein (bottom, Western blot with semiquantitative histograms). AIF was also upregulated upon wild-type TP53 overexpression. The TP53 target gene GADD45 was used as a control to show TP53 overexpression effect, and actin and GAPDH were used as internal control for RT-PCR and Western analysis, respectively. D, schematic representation summarizing data from the present study and earlier reports. Transcription of BNIP3 gene is activated by HIF-1α, whereas posttranscriptional regulation by miR145 controls BNIP3 mRNA translation. BNIP3 protein represses transcription of AIF (the transcription of which is also activated by TP53).
is regulated by HIF-1α, its overexpression in prostate cancer resulted from at least two fundamental molecular defects: overactivity of the transactivator HIF-1α at the transcriptional level and loss of the negative regulator miR145 at the post-transcriptional level (Fig. 4D).

**BNIP3 overexpression and its significance.** BNIP3 is a mitochondrial protein involved in cell death (11, 36) and is overexpressed in many cancers such as breast cancer, non-small cell lung cancer, glioblastoma multiforme, ovarian cancer, and uterine cervical and endometrial cancers (12, 14, 37–40), although it is underexpressed in several tumor types (e.g., tumors of the pancreas, stomach, and colon and rectum) through hypermethylation of CpG islands and histone H3 deacetylation (41–43). BNIP3 overexpression is associated with advanced stage in cervical cancer, poor prognosis in endometrial and non–small cell lung cancer, and tumor progression in breast ductal carcinoma in situ (14, 39, 40, 44).

Functionally, BNIP3 has been classified as a proapoptotic gene. Although overexpression of proapoptotic genes in cancer is well documented (e.g., caspase-3 and caspase-8, which are overexpressed in a variety of tumors; ref. 45), recent data indicate that they may have functions quite opposite to their conventionally assigned roles. For example, caspase-8, the most important initiator caspase in the extrinsic apoptotic pathways, possesses a surprising, catalytic activity-independent function of associating with focal adhesion molecule and calpain 2 to facilitate tumor cell migration and metastasis (45).

A similar scenario seems to be unfolding for BNIP3, which has been unexpectedly identified as a transcription repressor of the proapoptotic gene *AIF* (13). BNIP3 associates with PTB-associating splicing factor and histone deacetylase 1, and binds to the promoter of the *AIF* gene, hence repressing its expression and resulting in increased resistance to apoptosis. Thus, the dual nature of BNIP3 may explain its high levels in various solid tumors, including prostate cancer (present study) and glioblastoma multiforme (13), and its association with unfavorable outcome in prostate cancer (present study) and other tumors (14, 39, 40, 44).

**Mechanisms of miR145 downregulation.** An intriguing question is the cause(s) of miR145 downregulation in various tumors. Most recently, the transcription factors TP53 and OCT-4 have been shown to regulate transcription of miR145 (21, 34). In colorectal cancer cells, overexpression of TP53 or inhibition of the PI3K/AKT pathway by LY294002 (which upregulates TP53) results in the upregulation of miR145 and suppression of c-MYC (a miR145 target), through binding of TP53 to its response element in the miR145 promoter (21). Moreover, TP53 also participates in the maturation of miRNA (including miR145), by associating with DEAD-box RNA helicase p68 (DDX5) and interacting with the Drosha processing complex, facilitating the processing of primary to precursor miRNAs (46).

PC-3 cells are known for TP53 loss of heterozygosity and codon 138 mutation (resulting in frameshift and nonfunctioning, truncated TP53 protein that could be degraded quickly; refs. 22, 23). DU145 cells harbor mutations of codon 223 (CCT to CTT, Pro to Leu) and codon 274 (GTT to TTT, Val to Phe), also resulting in abnormal TP53 proteins (23, 47). Although LNCaP cells express wild-type TP53, it harbors mutation of CHK2 at codon 1160 (C to A, Thr to Asn), leading
to deficit of TP53 protein phosphorylation and activation (48). In prostate cancer tissue, about half harbor TP53 abnormalities. Thus, loss of functional TP53 may have contributed significantly to impaired miR145 expression, as TP53 is vital for both transcriptional activation and maturation of miR145. Because TP53 also regulates transcription of AIF (49), loss of TP53 function strikes double blows in prostate cancer by downregulating both miR145 (and consequent miR145-BNIP3 abnormalities) and AIF.

Alternative mechanisms for disrupted miR145-BNIP3 pairing, such as mutation, deletion, or epigenetic modifications of the miR145 gene and selective loss of BNIP3 3'-UTR, may exist; however, evidence is yet to be found. It is worth noting that inhibition of DNA methylation or histone deacetylation in lung adenocarcinoma does not result in miR145 upregulation (17). Our sequencing analysis did not show deletion or mutation of the miR145 gene in the studied prostate cancer cells, nor of the BNIP3 3'-UTR.

In summary, we identified BNIP3 as a functional target of miR145 and showed that the abnormalities of the miR145-BNIP3 pair might play major roles in prostate cancer tumorigenesis and progression. In light of recent elucidation of miR145 regulation by TP53 (21) and transcriptional repression of AIF by BNIP3 (13), the TP53-miR145-BNIP3-AIF axis may be an important part of the regulatory network derepressed in prostate cancer (Fig. 4D).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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