Tumor-suppressive microRNA-145 induces growth arrest by targeting SENP1 in human prostate cancer cells

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Key words
Gene regulation, miR-145, prostate cancer, SENP1, tumorigenesis

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Funding Information
National Natural Science Foundation of China; China Postdoctoral Science Foundation; Heilongjiang Postdoctoral Fund.

Received October 17, 2014; Revised January 23, 2015; Accepted January 28, 2015

Cancer Sci 106 (2015) 375–382
doi: 10.1111/cas.12626

Prostate cancer (PCa) prevails as the most commonly diagnosed malignancy in men and the third leading cause of cancer-related deaths in developed countries. Prostate cancer (PCa) prevails as the most commonly diagnosed malignancy in men and the third leading cause of cancer-related deaths in developed countries. One of the distinct characteristics of prostate cancer is overexpression of the small ubiquitin-like modifier (SUMO)-specific protease 1 (SENP1), and the upregulation of SENP1 contributes to the malignant progression and cell proliferation of PCa. Previous studies have shown that the expression of microRNA-145 (miRNA-145) was extensively deregulated in PCa cell lines and primary clinical prostate cancer samples. Independent target prediction methods have indicated that the 3′-untranslated region of SENP1 mRNA is a potential target of miR-145. Here we found that low expression of miR-145 was correlated with high expression of SENP1 in PCa cell line PC-3. The transient introduction of miR-145 caused cell cycle arrest in PC-3 cells, and the opposite effect was observed when miR-145 inhibitor was transfected. Further studies revealed that the SENP1 3′-untranslated region is a regulative target of miR-145 in vitro. MicroRNA-145 also suppressed tumor formation in vivo in nude mice. Taken together, miR-145 plays an important role in tumorigenesis of PCa through interfering SENP1.

Prostate cancer prevails as the most commonly diagnosed malignancy in men and the third leading cause of cancer-related deaths in developed countries. (1-3) This is mainly attributed to the extension of life expectancy and the widespread use of prostate-specific antigen for population screening in the North America and Europe. It has been reported that nearly 240,890 new cases of PCa were diagnosed in the USA in 2011, accounting for 29% of all newly diagnosed cancers in men. (4) Recently, the incidence of PCa has also risen sharply in China. (5) The development of human PCa is associated with genetic or epigenetic alterations, which result in abnormal gene expression profiles. Abnormalities of HPC1, androgen receptor (AR), and vitamin D receptor promote cancer cell growth. (6-10) Moreover, post-transcriptional regulation of gene expression by non-coding RNA, including miRNA, has recently attracted attention in relation to PCa development. (11-12)

Small ubiquitin-like modifiers’ conjugation to cellular proteins is a reversible post-translational modification that mediates the protein’s function, subcellular land/or expression. (13) The SENPs deconjugate modified proteins and thus are critical for maintaining the level of SUMOylated and un-SUMOylated substrates required for normal physiology. (14) Altered expression of SENPs is observed in several carcinomas. (15-16) One member of the SENP family, SENP1, can transform normal prostate epithelia to a dysplastic state and directly modulate several oncogenic pathways in prostate cells, including AR, c-Jun, and cyclin D1. (17) Assessment of tissue from human PCa patients indicates elevated mRNA levels of SENP1. For example, the work by Bawa-Khalfe et al. has shown that the expression level of SENP1 is upregulated in PCa cell lines. They also found that reducing SENP1 expression levels led to decrease of cellular viability. (18) Therefore, SENP1 plays critical roles in the development and progression of PCa, and SENP1 is a potential marker for PCa prognostics. In order to study the tumor biology of PCa, a number of human prostate cell lines have been established, among which DU145 and PC-3 cells lines have been extensively characterized and widely used for PCa research. (19)

MicroRNAs constitute a rapidly growing family of small (~22 nt) endogenous non-coding RNA molecules that regulate gene expression in a post-transcriptional manner through pairing with complementary nucleotide sequences in the 3′-UTR of target mRNAs. (16) Approximately half of the miRNA genes are located at fragile sites and genomic regions known to be frequently amplified or deleted in cancers. (17) Therefore, the abnormal expression of miRNAs has been suggested to play a critical role in prostate tumorigenesis. (18,19) Consequently, the study of miRNAs is prominent in cancer-related research nowadays, aiming to clarify their role during tumorigenesis and to examine their clinical efficacy.
Among the large number of studied miRNAs, miR-145 has a well-characterized tumor-suppressor regulatory role, which has been found to protect from cancer cell invasion and metastasis.\(^{(20)}\) Downregulated miR-145 levels have been documented in PCa.\(^{(21,22)}\) Inhibition of cell growth, in terms of cell cycle arrest and apoptosis, is promoted by miR-145 through the silencing of various target mRNAs.\(^{(23,24)}\) Among the predicted targets of miR-145, SOX9,\(^{(25)}\) insulin-like growth factor1 receptor,\(^{(26)}\) A disintegrin and metalloprotease 17,\(^{(27)}\) and L-dopa decarboxylase\(^{(28)}\) have already been found to have a crucial role during PCa establishment and progression, as well as to serve the clinical management of patients.

Considering the important roles in PCa development and progression of the two elements, SENP1 and miR-145, we hypothesize that SENP1 is a potential target of miR-145. To test the hypothesis, we investigated the expression profiles of SENP1 and miR-145 in PC-3 cells, and tested the interaction between SENP1 and miR-145 by luciferase assay. Our study revealed that miR-145 was significantly downregulated, and SENP1 upregulated, in PCa compared with normal epithelial prostate cells. The transient introduction of miR-145 inhibited cell proliferation in PC-3 cells, whereas the opposite effect was observed with transfection of miR-145 inhibitor. Moreover, miR-145 can bind to the 3'-UTR of SENP1 and reduce SENP1 expression by inhibiting translation and/or causing mRNA instability. Taken together, miR-145 is a prostate tumor suppressor that directly targets SENP1 in PC-3 cells.

**Materials and Methods**

**Cell lines and culture.** The PC-3 human prostate cancer cell line was obtained from the ATCC (Rockville, MD, USA), and the RWPE-1 human normal prostate epithelial cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The PC-3 cells were maintained in DMEM, and the RWPE-1 cells were maintained in RPMI-1640 medium, supplemented with 10% FBS (HyClone, Logan, Utah, USA), penicillin, and streptomycin. Cells were seeded in 24-well plates at 6 \(\times\) 10^4 cells per well for in vitro experiments. The cells were incubated in an atmosphere of 5% CO\(_2\) at 37°C.

**Transfection of miRNA.** The miR-145 mimics were designed and synthesized by Guangzhou RiboBio (Guangzhou, China). For transfection, the cells were plated on an antibiotic-free growth medium at 30–40% confluence approximately 24 h before transfection. RNA oligonucleotides were transfected at a final concentration of 50 nM, using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) according to the manufacturer’s protocol.

**Cell proliferation assay.** Cell proliferation was determined using the Cell Counting Kit-8 assay kit (Dojindo, Kumamoto, Japan) and the Cell Titer 96 assay kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The EdU incorporation assay was carried out according to the manufacturer’s instructions (Guangzhou RiboBio). Images were obtained and analyzed by High Content Imaging Pathway 855 (BD Biosciences, San Jose, CA USA). The fraction (%) of

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**Fig. 1.** Expression of has-miR-145-5p (miR-145) and SENP1 in prostate cancer (PCa) and normal prostate epithelial cell lines. miR-145 expression (a) and SENP1 (b) expression in different cell lines was analyzed by real-time PCR and standardized against the expression of U6 small nuclear RNA, which was used as an endogenous control. (c) Expression of SENP1 in PCa patients was quantified by real-time PCR. (d) Protein expression of SENP1 in different PCa cell lines. The data are presented as the average of triplicate values. Error bars, SEM. *\(P < 0.05\), **\(P < 0.01\), compared with the negative control.
EdU-positive cells was calculated as (EdU add-in cells/Hoechst stained cells) × 100.

**Cell cycle assay.** PC-3 cells were transfected after 24 h, as previously described. Seventy-two hours later, the cells were collected and washed twice with PBS, resuspended in 300 µL of PBS, and fixed by adding 700 µL of 100% ethanol at 4°C for 24–48 h. The fixed cells were rinsed three times with PBS and stained with a propidium iodide solution containing 100 µg/mL propidium iodide and 50 µg/mL RNase (Sigma, Shanghai, China) in PBS at 37°C for 30 min in the dark. Stained cells were passed through a nylon mesh sieve to remove cell clumps and then were analyzed by flow cytometry (BD, San Jose, USA). Data were collected and analyzed by the CellQuest (http://www.bdbiosciences.com/video/Cell-Quest.zip) and ModFit Lt (http://www.zedload.com/modfit-lt-3.2-trial-version-crack-serial-download.html) software.

**RNA extraction, reverse transcription, and quantitative PCR.** Total RNA, including miRNA, was extracted using TRIzol (Invitrogen), according to the manufacturer’s instructions. RNA was synthesized into cDNA using M-MLV reverse transcriptase (Promega) in a 25-µL volume. The primer sequences for miR-145 amplification were: hsa-miR-145 F, ACACTCCAGCTGGGGTCCAGTTTTCCCAGGAA and R, CTCATCTGGTGTGGTCCA. The primers for SENP1 were: F, CACGAGATGAATGGAAGTGA and R, CCGGAATTATGGCATGTGT. The U6 gene was used as internal reference (29): F, CTCGCTTCGGCAGCACA and R, AACGCTTCACGAATTTGCGT. Real-time PCR was carried out using SYBR Green mix (TaKaRa, Shanghai, China) in a 20 µL reaction volume on an MJ Opticon Monitor Chromo 4 instrument (Bio-Rad, Hercules, CA, USA), using the following protocol: 95°C for 5 min and then 35 cycles of 95°C for 10 s, 60°C for 20 s, and 70°C for 10 s. The expression of each miRNA was normalized to U6 small nuclear RNA and calculated using the 2–ΔΔCt method.

**Western blot analysis.** Cellular proteins were separated in 10% SDS–polyacrylamide gels, electroblotted onto an Immobilon-P transfer PVDF membrane (Millipore, Temecula, CA, USA), detected with a rabbit anti-human SENP1 mAb (04-453, 1:1000; Millipore), a rabbit anti-Ki67 polyclonal antibody (AB9260, 1:500; Millipore) or a rabbit anti-human-GAPDH antibody (AB9260, 1:500; Millipore) and then visualized by a commercial ECL kit (Beyotime, Beijing, China).

**Luciferase reporter assay.** To create a luciferase reporter plasmid, the 3′-UTR fragment containing putative binding sites for miR-145 was PCR-amplified from the genomic DNA of PC-3 cells. The PCR products were gel-purified (Dongsheng Biotech, Shanghai, China), digested (New England Biolabs, Ipswich, MA, USA), and ligased (Takara, Tokyo, Japan) into the digested psiCheck-2 plasmid (Promega) between the XhoI and NotI sites. The SENP1 3′-UTR-targeted site mutations were

### Fig. 2

Has-miR-145-5p (miR-145) inhibits cell growth and SENP1 promotes cell proliferation in PC-3 prostate cancer cells. (a, b) PC-3 cells were transfected with 50 nM miRNA mimics, SENP1, si-SENP1, or negative control (NC; empty vector) in 96-well plates on day 0. The effect of miR-145 and SENP1 in the PC-3 cells was analyzed using the 5′-ethyl-2′-deoxyuridine (EdU) incorporation assay. (c) Cell cycle profile changes were assessed by flow cytometry using propidium iodide staining to measure the DNA content. The data are presented as the average of triplicate values. Error bars SEM. *P < 0.05, **P < 0.01, which was considered to be significantly different compared with NC.
generated using the KOD-plus mutagenesis kit (Toyobo, Osaka, Japan), according to the manufacturer’s protocol.

PC-3 cells were plated in a 96-well plate and then cotransfected with 200 ng/µL plasmid and 50 nM miR-145 or NC. Forty-eight hours after cotransfection, luciferase activity was detected by the Dual-Glo luciferase assay kit (Promega). The transfections were carried out in duplicate and repeated three times.

Tumor formation in BALB/c nude mice. All experimental procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised 1996); all experimental procedures involving humans were in accordance with the sixth version of the Declaration of Helsinki (revised 2008). Experiments were carried out in compliance with the institutional ethical guidelines for animal and human experimentation. The participants all gave signed informed consent. PC-3 cells were pretreated with the miR-145 mimics 184 (50 nM) or NC. After 24 h of transfection, the cells were suspended in 100 µL PBS at a concentration of 4 x 10^6 cells/mL and injected into either flank of the same BALB/C female athymic nude mouse at 5–6 weeks of age (10 mice for each group, i.e., n = 10). The tumor size was monitored by measuring the length (L) and width (W) with calipers, and the volumes were calculated using the formula: (L x W^2) x 0.5.

Statistical analysis. All statistical analyses were carried out with srs 19.0 software (SPSS, Chicago, IL, USA). The data are presented as the mean ± SD from three separate experiments. Statistical significance was determined by paired or unpaired Student’s t-test in cases of standardized expression data. Differences were considered statistically significant at P < 0.05.

Results

Reduced expression of miR-145 and elevated expression of SENP1 in PC-3 cells. Recently, we detected miR-145 expression profiles in prostate cancer cell line DU145, PC-3, and LNCaP. Compared with the non-immortalized immortalized prostate cell RWPE-1, we found that all miR-145 was downregulated in these three PCa cell lines (Fig. 1a). The differences in miR-145 level between PCa cells and RWPE-1 cells were significant (P < 0.05). More importantly, DU145 and PC-3, hormone-independent PCa cells, appeared to have lower miR-145 expression, which implied that decreased expression of miR-145 might contribute to the malignant progression of PCa.

The transcriptional and translational expression of SENP1 was also examined in the three cell lines by quantitative RT-PCR and compared with RWPE-1 (Fig. 1b). All three PCa cells overexpressed SENP1 at both mRNA and protein levels. More importantly, DU145 and PC-3, PCa cells with high metastatic potential, appeared to have stronger SENP1 expression, 2–3-fold higher than that in RWPE-1. This implied that SENP1 overexpression might contribute to the malignant progression of PCa. To gain insight into the detailed biological role of SENP1 in human prostate carcinogenesis, we analyzed the expression of SENP1 in four human PCa and their paired paratumor tissues. The clinical characteristics of patients are listed in Table S1. Compared with the paired paratumor tissues, significant upregulation of SENP1 was observed in the tumor tissues (P < 0.05, Fig. 1c). From these data, we hypothesize that the expression of SENP1 is associated with miR-145.

PC-3 cell proliferation inhibited by miR-145 and promoted by SENP1. Next, we evaluated the effect of miR-145 and SENP1 on the growth of PC-3 cells. As shown in Figure 2(a), miR-145 or si-SENP1 introduction caused a remarkable inhibition of cell growth in PC-3 cells relative to NC, whereas an opposite observation was found when SENP1 was introduced into PC-3 cells. The EdU incorporation percentage also revealed that the growth of PC-3 was significantly inhibited by miR-145 relative to NC (P < 0.05, Fig. 2b). The EdU cell proliferation assay determined that miR-145 and si-SENP1 suppressed the entry of PC-3 cells into S phase. To further characterize the effect of miR-145 on the cell cycle, we analyzed the cell cycle distribution in transfected cells by flow cytometry. The miR-145 mimics and si-SENP1 caused a higher G0/G1 arrest in PC-3 cells (Fig. 2c). The suppressive effect could be reversed by the introduction of SENP1; more than 90% of cells were Edu-positive in this group and the cells in S phase increased relative to NC or the other two groups (P < 0.05). These results suggest that miR-145 introduction suppresses cell growth.
proliferation and SENP1 promotes cell proliferation in PC-3, and both of them regulate cell proliferation during G1 to S phase of the cell cycle. These data further prove the hypothesis that expression of SENP1 is associated with miR-145.

SENP1 is a direct target of miR-145 in PC-3 cells. To determine the molecular mechanism by which miR-145 induces cell growth arrest and senescence, we used three open-target prediction programs (picTar, TargetScan, and miRanda) to predict the targets of miR-145. In order to confirm our previous hypothesis, we compared the sequences of 3′-UTR of SENP1 with miR-145. The 3′-UTR of SENP1 mRNA contained a complementary site for the seed region of miR-145 (Fig. 3a), and the highly matched bases attract our attention to investigate whether SENP1 is a putative target of miR-145. SENP1 is a notably attractive candidate because it plays important roles in the development and progression of PCA.

To determine whether SENP1 is the direct target gene for miR-145, a dual-luciferase reporter system was used. The luciferase reporter assay indicated that the luciferase activity of the reporter containing the SENP1 gene’s wild-type 3′-UTR decreased (50%) following treatment with miR-145 mimics. By contrast, the inhibitory effect of the miR-145 mimics was abolished in the mutated construct (Fig. 3b). Moreover, the luciferase activity decreased by miR-145 was inhibited when SENP1 overexpression was introduced. The result indicates that miR-145 most likely suppresses gene expression through miR-145 binding sites in the 3′-UTR of SENP1. In addition, quantitative RT-PCR and Western blot analysis revealed that the expression of SENP1 mRNA and protein was inhibited by treatment with miR-145 mimics in PC-3 cells (Fig. 3c,d). Taken together, these data suggest that miR-145 reduces SENP1 expression by inhibiting translation and/or causing mRNA instability.

Overexpression of miR-145 can inhibit proliferation of PC-3 promoted by SENP1. To determine whether miR-145 mediates its growth-repressing effects primarily through SENP1, and whether SENP1 reverses the inhibition of cell growth caused by miR-145, PC-3 cells were cotransfected with miR-145 mimics and pEZ-M02-SENP, which encoded the entire SENP1 coding sequence, by measuring cell proliferation rate and inhibition rate. When only SENP1 was overexpressed in PC-3 cells, the OD_{450} values in the SENP1 group was significantly higher than the control groups (PC-3 cells and empty vector group, \(P < 0.05\)), which meant that cells proliferated more quickly than the control group. However, when miR-145 was cotransfected with SENP1, the cell proliferation caused by SENP1 was significantly inhibited by miR-145 from the third day after cotransfection (\(P < 0.05\), Fig. 4a). Next, the proliferation and inhibition rates of transfected PC-3 cells were calculated to further demonstrate the growth-suppressing effect of miR-145. In Figure 4(b,c), the introduction of miR-145 to PC-3 cells obviously decreased the proliferation rate promoted by SENP1. Therefore, miR-145 mediated its growth-repressing effects primarily through SENP1.

SENP1 promotes tumor growth in vivo. To confirm the tumor suppressor role of miR-145, we established a BALB/c nude mouse xenograft model using PC-3 cells. The PC-3 cells were pretransfected with miR-145, then injected into female BALB/c nude mice to form tumors. The tumor volume was measured every 2 days until day 28. The tumor volume of the PC-3 cells treated with miR-145 mimics was significantly reduced relative to PC-3 cell group (Fig. 5a,b). This result indicates that miR-145 introduction significantly inhibits the tumorigenicity of PC-3 cells in the nude mouse xenograft model. Representa-

tive photographs of PCA tissues formed in nude mice showed the increased expression of SENP1 in tumors would promote the tumorigenesis, which was reversed in miR-145 treated cells (Fig. 5c). In order to confirm whether SENP1 expression was suppressed by transfected miR-145 mimics in the formed tumors, we examined the expression of SENP1 in formed tumors at day 30 (Fig. 5d). The tumors formed by cells transfected with miR145 showed a sharp decrease expression of SENP1 and Ki67, which suggested that miR145 indeed inhibited the proliferation of cancer cells.

Discussion

Growing evidence indicates that deregulation of miRNAs contributes to human carcinogenesis.\(^{(30,31)}\) One major tumor suppressor miRNA, miR145, is downregulated in such neoplasms as colorectal, mammary, ovarian, and B-cell tumors.\(^{(32,33)}\) Recent reports indicated that miR145 is also among the down-regulated miRNAs in prostate cancer.\(^{(34)}\) In some tumors, downregulation of miR145 is correlated with tumor size, stage, proliferative activity, or poorer prognosis.\(^{(35,36)}\) Gradual decrease of miR145 is observed in mammary neoplasia,\(^{(37)}\) whereas artificial overexpression of miR145 inhibits cell growth and tumor formation.\(^{(36,38)}\) Downregulation of miR145
in various cancers has made it one of the most noticeable tumor suppressor miRNAs.\(^{39}\)

Identification of miRNA targets is one of the most important aspects in understanding the mechanisms by which miRNAs control cell behavior. Several genes, including \(IRS-1\), \(OCT4\), \(SOX2\), \(KLF4\), \(C-MYC\), and \(RTKN\) (rhotekin), have been identified as miR145 target genes.\(^{38,40–42}\) In our study, we found that miR-145 inhibits cell proliferation by repressing the expression of \(SENP1\), which is a novel target for miR-145 in PCa cells. Our results suggest that miR-145 plays an important role in the inhibition of tumorigenesis of PCa. Our current results indicate that miR-145 is downregulated in common PCa cell lines compared with the corresponding, non-cancerous prostate epithelia cells. However, a reverse expression profile of \(SENP1\) is observed in PCa cell lines. These two genes were reportedly associated with the growth of PCa, and miR-145 or \(SENP1\) was an important diagnostic marker of PCa. In addition to the expression profile, we also found that ectopic miR-145 inhibited cell proliferation in PCa cell lines, whereas \(SENP1\) could efficiently promote cell proliferation. Taken these results, we hypothesized that there would be some relationship between these two genes.

To explore the mechanism underlying the suppression of prostate cancer cell growth, we determined that \(SENP1\) was a direct target of miR-145 in PC-3 by a luciferase reporter system. A family of human \(SENP\)s can selectively deconjugate SUMOylated proteins and hence dictate SUMO dynamics.\(^{43}\) Recently, we reported that \(SENP1\) plays a prominent role in the regulation of the AR-dependent transcription.\(^{44}\) Androgen receptor activity is modulated by \(SENP1\) in PCa cells; overexpressing \(SENP1\) increases AR transcriptional activity by de-SUMOylation of the co-regulatory protein, histone deacetylase 1.\(^{44}\) Reduction of endogenous \(SENP1\) in these cells, using interfering RNA directed specifically against \(SENP1\), significantly decreases expression of the AR-regulated prostate-specific antigen gene. Similarly, \(SENP1\) moderates the expression of the cell cycle regulator, cyclin D1; diminishing \(SENP1\) in PCa cells decreases cyclin D1 levels.\(^{12}\) Previous studies indicate that enhanced expression of cyclin D1 is readily observed in advanced PCa and contributes to PCa progression.\(^{45,46}\) Therefore, the expression of \(SENP1\) in PCa cells modulates major factors in PCa progression. In our study, the EdU cell proliferation assay and the cell cycle assay revealed that miR-145 introduction induced a quiescent phenotype in PC-3 cells. This phenotype resembles
those in previous studies that inhibited SENP1 expression. Therefore, our study suggests that SENP1 downregulation by miR-145 overexpression may be a key event in the induction of cellular quiescence. This was confirmed by cotransfection assay in our study. The proliferation of PC-3 cells promoted by SENP1 could be clearly inhibited by the introduction of miR-145. Our findings confirmed that SENP1 is a direct target of miR-145 in PCa. Therefore, miR-145 might function as a tumor suppressor by targeting multiple oncogenes, and miR-145 reactivation by pharmacologic agents might have therapeutic value in human cancer.

In summary, we have found that miR-145 acts as a tumor suppressor and is mostly downregulated in PCa cell lines. Low-level expression of miR-145 is correlated with cancer relapse and death, and miR-145 suppresses tumor cell growth in vitro and tumorigenicity in vivo. Introduction of miR-145 into PCa cell lines leads to inhibition of cell proliferation by directly targeting SENP1. Hence, our data suggest that miR-145 may have prognostic or therapeutic value for the future management of PCa patients.

Acknowledgments
This work was financially supported by the National Natural Science Foundation of China (Grant No. 81402123), a General Financial Grant from the China Postdoctoral Science Foundation (Grant No. 2012MS10995), a Special Financial Grant from the China Postdoctoral Science Foundation (Grant No. 2014T70369), and the Heilongjiang Postdoctoral Fund (Grant No. LBH-Z11068).

Disclosure Statement
The authors have no conflict of interest.

Abbreviations
AR androgen receptor
EdU S′-ethynyl-2′-deoxyuridine
miR-145 has-miR-145-5p
miRNA microRNA
NC negative control
PCa prostate cancer
SENP SUMO proteases
SUMO small ubiquitin-like modifier
UTR untranslated region

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Clinical characteristics of patients with prostate cancer (n = 4)