Heat-shock Protein HSPB1 Attenuates MicroRNA miR-1 Expression Thereby Restoring Oncogenic Pathways in Prostate Cancer Cells

MATTHIAS B. STOPE1, CHRISTIAN STENDER1, TINA SCHUBERT1, STEFANIE PETERS1, MARTIN WEISS1, PATRICK ZIEGLER2, UWE ZIMMERMANN1, REINHARD WALThER3 and MARTIN BURCHARDT1

Materials and Methods

Cell culture. PCa cell lines LNCaP and PC-3 (Cell Lines Service, Heidelberg, Germany) were propagated in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1%
penicillin/streptomycin (PAN Biotech, Aidenbach, Germany) in an atmosphere with 5% CO₂ and at 37°C. PC-3-HSBP1 cells stably-overexpressing U6 RNA expression levels. Data are given as the mean±SD, statistically analyzed by Student's t-test. *p≤0.05; **p≤0.01; *** p≤0.001.

Transfection experiments. One day prior to transfection, cells were plated into 6-well (150,000 cells/well) or 24-well (30,000 cells/well) cell culture plates coated with 0.01% poly-L-lysine (Sigma-Aldrich, Deisenhofen, Germany) for 10 min. For overexpression of miR-1 and HSPB1, 1 μg DNA (24-well) and 3 μg DNA (6-well) per well were transferred into cells using Lipofectamin 2000 reagent (Invitrogen, Karlsruhe, Germany). miR-1-specific inhibitor molecules Anti-hsa-miR-1 miScript miRNA Inhibitor (Qiagen, Hilden, Germany) were provided by C. Kubisch (Munich, Germany). Empty vectors pcDNA3.1 (Invitrogen) and pSuperior (OligoEngine, Seattle, WA, USA) were used for control transfections.

Cloning of the miR-1 mimicking expression vector pmIR-1. A DNA plasmid encoding for the cDNA sequence of mature miR-1 mimicking RNA (pmiR-1) was constructed using the pSuperior system from OligoEngine. Two oligonucleotides (miR-1 forward 5' - GATCCCCGGGTGAAATGATGTATTTCAAGGAGATACAACTTCTTTACATTCGATATTTCAAAGTTAATT-3' and miR-1 reverse 5'-TCGATAAAATTGGATGATGAATGTTTTGATTTTAACTTCTTTACATTCGATTCACTTACAGG-3') were hybridized by a temperature gradient from 95°C to 4°C for 40 min. Due to the asymmetrical design of both complementary oligonucleotides, the hybridization products formed defined single-stranded 5’ overhangs, which became ligated into the BglII/Xhol (Thermo Scientific, Waltham, MA, USA) double-digested pSuperior vector. After ligation (T4 DNA Ligase; Thermo Scientific), positively-selected clones were verified by restriction analysis and sequencing.

MicroRNA quantification by reverse transcription and real-time polymerase chain reaction (qRT-PCR). Total small RNA was extracted using the mirPremier microRNA Isolation Kit (Sigma-Aldrich) and 2 μg of total microRNA were applied in reverse transcription by using the miScript II RT Kit (Qiagen). Subsequently, quantification was performed by real-time PCR (miScript SYBR Green PCR Kit; Qiagen) with the CFX96 Real-Time PCR Detection System (BioRad) and the primer HS_miR-1 primer Assay (Qiagen). For quantification, miR-1 signals were standardized to U6 RNA (HS_RNU6-2_1 miScript Primer Assay; Qiagen) as reference.
mRNA quantification by qRT-PCR. Total RNA was prepared by peqGOLD TriFast (Peqlab, Erlangen, Germany) according to the supplier’s descriptions and quantified using a Nanodrop ND-2000 UV/vis spectrophotometer (Peqlab). Reverse transcription was carried out with 1 μg of total RNA, the Superscript III First-Strand Synthesis System (Invitrogen) and an oligo dT primer. Real-time PCR was carried out using a fluorescence dye (SensiMix SYBR KR; Bioline, London, UK) and the CFX96 Real-Time PCR Detection System (BioRad) with the following target-specific primer pairs: AR forward: 5’-TGCCCTGATCTGTGGAGATGA-3’, AR reverse: 5’-CAAGAGCGACAAGATGGACA-3’, TGFβ1 forward: 5’-GCCCTGAGCA CCAACTATTG-3’, TGFβ1 reverse: 5’-CTGTGCTGTCATCATCCACC-3’, kallikrein-3 (KLK3) forward: 5’-CGGAGAGCTGTGTC ACCAT-3’, KLK3 reverse: 5’-GTGCAGCAACCAATCCACGTCT-3’, and ribosomal protein large P0 (RPLP0) forward: 5’-CAATGG CAGCATCTCAACC-3’, RPLP0 reverse: 5’-ACTCTTCCTTGGC TTCAACC-3’ as reference gene.

Generation of LNCaP cells stably-overexpressing miR-1-mimicking RNA. LNCaP cells were transfected with the vector pmiR-1 as described above, passed once per week and selected with 0.3 μg/ml puromycin (Invitrogen). Cell clones stably overexpressing miR-1-mimicking RNA molecules were identified by qRT-PCR and compared to maternal LNCaP cells. Subsequent cultivation of LNCaP-miR-1 cells was performed in the presence of 0.3 μg/ml puromycin.

Proliferation assay. Cellular proliferation was examined by cell counting utilizing a CASY Cell Counter and Analyzer Model TT (Roche Applied Science, Mannheim, Germany). Therefore, 30,000 cells/well were plated in 24-well cell culture plates and treated for 96 h or 144 h. At indicated time points, adherent cells were detached by trypsin treatment, suspended in 10 ml CASYton solution (Roche Applied Science), and the number of living cells was determined in 400 μl cell suspension in triplicates.

Statistical analysis. Results of at least three independent experiments were statistically analyzed, using the unpaired Student’s t-test, and expressed as the mean±SD compared to control cells. Differences at ps0.05 were considered significant.

Results

HSPB1 diminishes microRNA mir-1 expression in PCa cells. The basal expression of miR-1 in PCa cell lines LNCaP and PC-3 was negatively correlated to the intracellular HSPB1 concentration. The higher the level of HSPB1 protein in LNCaP cells (Figure 1A), the lower the expression of miR-1 was (defined as 1.0; Figure 1B), whereas the low abundance of HSPB1 protein in PC-3 cells (Figure 1A) related to a significantly elevated level of miR-1 (71.9-fold, p<0.0014; Figure 1B). From there, we assumed a regulatory connection between HSPB1 and miR-1 and we conducted further experiments to validate our hypothesis.

As shown by qRT-PCR, stable overexpression of HSPB1 (Figure 1A) reduced high expression levels of miR-1 from 71.9-fold to 13.2-fold (p=0.0028; Figure 1B) compared to LNCaP cells. These observations were additionally confirmed by transient transfections, with transient up-regulation of miR-1 overexpression attenuates PCa cell proliferation. These data prompted us to study whether the identified suppression of miR-1 may be part of HSPB1-driven resistance pathways potentially counteracting the antiproliferative efficacy of cancer drugs. Therefore, the DNA plasmid pmiR-1 encoding for an miR-1 cDNA sequence was constructed, showing greatly enhanced overexpression of miR-1-mimicking RNA, resulting in a 810.7-fold increase of miR-1 72 h after transfection (p<0.0455; Figure 2A). miR-1 analysis after DNase treatment of microRNA preparations, as well as control reactions using the DNA plasmid pmiR-1 as PCR template were negative for miR-1 signals (data not shown). To assess knock-down of miR-1, a commercially available miR-1-specific inhibitor was used, which led to significant and concentration-dependent suppression of miR-1 expression of 2.2-fold at 10 nM (p<0.0001), 6.3-fold at 20 nM (p<0.0001), and 26.1-fold at 40 nM (p<0.0001), as shown in Figure 2B. Following miR-1 inhibition, experiments were carried out applying an miR-1 inhibitor at a concentration of 40 nM.

HSPB1 (Figure 1A) revealing a clear reduction of miR-1 level from 63.9-fold (Figure 1B) to 15.3-fold (p=0.0001; Figure 1B) compared to basal miR-1 expression in LNCaP cells.

miR-1 overexpression attenuates PCa cell proliferation. These data prompted us to study whether the identified suppression of miR-1 may be part of HSPB1-driven resistance pathways potentially counteracting the antiproliferative efficacy of cancer drugs. Therefore, the DNA plasmid pmiR-1 encoding for an miR-1 cDNA sequence was constructed, showing greatly enhanced overexpression of miR-1-mimicking RNA, resulting in a 810.7-fold increase of miR-1 72 h after transfection (p<0.0455; Figure 2A). miR-1 analysis after DNase treatment of microRNA preparations, as well as control reactions using the DNA plasmid pmiR-1 as PCR template were negative for miR-1 signals (data not shown). To assess knock-down of miR-1, a commercially available miR-1-specific inhibitor was used, which led to significant and concentration-dependent suppression of miR-1 expression of 2.2-fold at 10 nM (p<0.0001), 6.3-fold at 20 nM (p<0.0001), and 26.1-fold at 40 nM (p<0.0001), as shown in Figure 2B. Following miR-1 inhibition, experiments were carried out applying an miR-1 inhibitor at a concentration of 40 nM.

In several cancer entities, miR-1 properties are linked to cellular growth inhibition and therefore we conducted growth kinetics assays with cells overexpressing miR-1 molecules. In LNCaP cells transiently transfected with pmiR-1, elevated miR-1 levels were confirmed by qRT-PCR (data not shown). Cellular
growth analysis tended to show a 2.0-fold but statistically insignificant decrease of cell numbers within 96 h (p=0.1454; Figure 3A). These data were confirmed by experiments using LNCaP cells stably overexpressing miR-1 (LNCaP-miR-1) which exhibited an intense increase of basal miR-1 expression compared to maternal LNCaP cells (278-fold, p=0.0003; Figure 3B). Subsequent growth kinetic assays showed proliferative characteristics comparable to those on transient miR-1 transfection, with a 7.1-fold reduction of cellular growth after 144 h following miR-1 expression (p=0.0243; Figure 3C).

miR-1-driven growth inhibition is obligated by AR and TGFB pathway suppression. From the blockade of cellular growth processes, we concluded that miR-1 has selective effects on proliferative pathways in PCa. By modulating miR-1 expression, we found the AR to be suppressed by miR-1 on the mRNA (2.0-fold, p=0.0364; Figure 4A) and protein (1.5-fold, p=0.0047; Figure 4B) levels, as well as its transcriptional activity (7.0-fold, p<0.0001; Figure 4C), the latter assessed as reduced transcription of the AR target gene KLK3. Besides down-regulating proliferative AR signals, we additionally identified TGFB1 signaling to be targeted by miR-1. Transient overexpression of miR-1 in LNCaP cells expressing a low level of miR-1 led to significant reduction of TGFB1 (2.0-fold, p=0.0253; Figure 4D), whereas the inhibition of miR-1 expression in PC-3 cells expressing high levels of miR-1 caused an increase of transcriptional rates of TGFB1 mRNA (2.7-fold, p=0.0043; Figure 4E).

Discussion

Our current findings significantly expand the understanding over HSP-driven chemoresistance and qualify the HSPB1-regulated microRNA miR-1 as a target structure for development of DNA-based drugs and furthermore as an appropriate molecule for indirect targeting by HSPB1 inhibition. We found evidence that HSPB1 regulates miR-1, which for the first time provides evidence that an HSP operates as a regulator of microRNA expression. Our observations were supported by histological studies which showed correlations of elevated HSPB1 expression (11, 12) and diminished miR-1 levels (13) in primary PCa tissue.

Since 2007, approximately 80 microRNA species have been identified and partially distinguished by pro- and anti-oncogenic properties in PCa progression (14-16). One of these is miR-1, however, relatively little is known about the cellular role of miR-1 in PCa. Formerly, miR-1 was thought to be specific for muscle cells in skeletal muscle and heart tissue, with they being very low or nearly undetectable levels in other organs (17). Even though reduced miR-1 expression in PCa tissue samples was described in 2008 (13), to our knowledge only four publications exist specifying miR-1 functionality in PCa cells. Thus, miR-1 was linked to decreased proliferation, enhanced motility and epithelial-mesenchymal transition of PCa cells (18-20), indicating miR-1 functionality as a tumor suppressor and corroborate the findings of the data presented here.
Very interestingly, based on AR and TGFB1 analysis after miR-1 modulation, our study shows that miR-1 is an inhibitor of AR and TGFB1 cascades. Both signaling pathways are known key regulators of oncogenesis and tumor proliferation, and thus, promote PCa progression (21, 22). From this it follows that anti-therapeutic activity of frequently upregulated HSPB1 suppresses the anticancer properties of miR-1 and subsequently re-activates tumor-promoting cascades of AR and TGFB1 signaling. Overexpression of miR-1-mimicking RNA in LNCaP cells led to down-regulation of AR expression (Figure 4A to C), which may contribute, at least, partly to HSPB1-mediated induction of AR expression (7). Interestingly, inhibiting miR-1 synthesis in AR-negative PC-3 cells caused an increase of AR mRNA, however, AR protein re-expression was not detectable (data not shown). Previous experiments of our group demonstrated that miR-1 also diminishes biosynthesis of shortened, constitutively active AR isoforms (23). Besides AR signaling, miR-1 suppresses the multifunctional growth factor TGFB1 and, to our knowledge, we identified for the first time HSPB1-driven control of TGFB1 signal transduction in PCa cells. This is very important due to the dual role of TGFB1 signaling in cancer cells. The multi-functional nature of TGFB1 is reflected in its opposing pro-oncogenic effects, e.g., proliferation, metastasis, immunosuppression, and remodeling of the extracellular matrix, as well as anti-oncogenic effects on cellular growth inhibition and induction of apoptosis (21, 24). Moreover, it has been shown that TGFB1 pathways are linked to AR signaling (25) and therefore may facilitate ligand-independent growth of castration-resistant PCa. Thus, HSPB1-driven suppression of miR-1 restores AR and TGFB1 signaling, which may represent an important step in enabling PCa cell growth in a low androgen environment and in the presence of anticancer drugs.

In conclusion, our findings improve the understanding of survival mechanisms in drug-resistant PCa cells. Besides inhibition of apoptotic pathways (26, 27), chemotherapy-induced HSPB1 expression initiates the inhibition of miR-1 and subsequently the restoration of pro-oncogenic AR and TGFB1 signals. Moreover, our data point to promising new targets for alternative therapy approaches: HSPB1 inhibition and miR-1-mimicking nucleic acid compounds may reduce HSPB1-miR-1-driven treatment resistance in PCa therapy.

Acknowledgements

The Authors thank Anne Brandenburg and Katja Wittig for excellent technical assistance.
References


Received March 18, 2014
Revised May 16, 2014
Accepted May 19, 2014