Survival in high-risk prostate cancer patients is predicted by mir-221, which regulates proliferation, apoptosis and invasion of prostate cancer cells by inhibiting IRF2 and SOCS3

Burkhard Kneitz, Markus Krebs, Charis Kalogirou, Maria Schubert, Steven Joniau, Hein van Poppel, Evelyne Lerut, Susanne Kneitz, Claus Jürgen Scholz, Philipp Ströbel, Manfred Gessler

Hubertus Riedmiller, Martin Spahn

Department of Urology and Paediatric Urology, University Hospital Wuerzburg, Würzburg, Germany
Department of Urology, University Hospital Bern, Inselspital, Bern, Switzerland
Department of Urology, University Hospital Leuven, Leuven, Belgium
Department of Pathology, University Hospital Leuven, Leuven, Belgium
Physiological Chemistry I, Biocenter, University of Wuerzburg, Wuerzburg, Germany
IZKF Laboratory for Microarray Applications, University Hospital of Wuerzburg, Wuerzburg, Germany
Department of Pathology, University Hospital Goettingen, Goettingen, Germany
Developmental Biochemistry, Biocenter, University of Wuerzburg, Wuerzburg, Germany.

Address correspondence to:
Burkhard Kneitz: Department of Urology and Paediatric Urology, University Hospital Wuerzburg, University of Wuerzburg Oberdärrbacher Str.8 D-97080 Wuerzburg, Germany
e-mail: Kneitz_B@klinik.uni-wuerzburg.de
Tel.: +49 921 201 32700 Fax: +49 931 201 32719

Martin Spahn: Department of Urology, University of Bern
Anna Seiler-Haus
CH-3010 Bern, Switzerland
e-mail: martin.spahn@insel.ch
Tel.: +41 31 632 3620 Fax:

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Abstract:

A lack of reliably informative biomarkers to distinguish indolent and lethal prostate cancer (PCa) is one reason this disease is overtreated. miR-221 has been suggested as a biomarker in high risk PCa, but there is insufficient evidence of its potential utility. Here we report that miR-221 is an independent predictor for cancer-related death, extending and validating earlier findings. By mechanistic investigations we showed that miR-221 regulates cell growth, invasiveness and apoptosis in PCa at least partially via STAT1/STAT3 mediated activation of the JAK/STAT signalling pathway. miR-221 directly inhibits the expression of SOCS3 and IRF2, two oncogenes that negatively regulate this signalling pathway. miR-221 expression sensitized PCa cells for IFN-γ mediated growth inhibition. Our findings suggest that miR-221 offers a novel prognostic biomarker and therapeutic target in high-risk PCa.
Introduction:

In Europe, the number of newly diagnosed prostate cancer (PCa) cases per year increased from 145,000 in 1996 to 345,000 in 2006. Despite this dramatic increase, the number of deaths attributed to the disease over the same time period remained almost unchanged (75,000 in 1996 vs. 68,000 in 2006) (1, 2). The current inability to accurately distinguish risk of life-threatening, aggressive PCa from indolent cases contributes to the dilemma. The identification of factors that are specifically associated with lethal PCa is urgently needed to reduce overtreatment, as well as to develop more effective targeted therapies.

Several potential prognostic markers have been identified and there is a plethora of promising biomarkers including Kallikrein-2, p53, Ki67, PTEN-loss, CCP-scores and ETS gene fusions (3). But, none of these markers has had made it into clinical use yet. This is mainly due to tumor heterogeneity and the patient cohorts analyzed (4). One possibility to optimize a biomarker screening strategy is using high-risk PCa-cohorts. 20-35% of all newly diagnosed PCas are classified as high-risk PCa (PSA >20ng/ml, biopsy Gleason Score (GS) ≥8, clinical stage T3/4) (5). Up to 30% of these men will develop metastasis and finally die of their disease (6-8). Based on these relatively high event rates, if compared to low-/intermediate risk study groups, high-risk PCa represents a good cohort to validate pre-existing biomarkers predicting clinical failure (CF) and cancer related death (CRD).

Micro-RNAs, small non-coding RNA molecules, play pivotal roles in carcinogenesis and can function as tumor suppressor- or oncogene-miRs (9). Extensive evidence has indicated that miR-221 dysregulation plays an important role in PCa development and progression. Several studies showed that miR-221 is one of the most strongly and frequently down-regulated miRNAs in primary-PCa (10-12). Furthermore we demonstrated that miR-221 is progressively downregulated in aggressive PCa, lymph node-metastasis and has potential as a biomarker predicting CF in high-risk PCa (13). In contradiction to the observed miR-221 downregulation in PCa, miR-221 overexpression has been reported for various other tumor...
types such as cancer of lung, bladder, thyroid, breast, liver or pancreas (14-16). Overexpression of miR-221 in cell lines derived from the latter tumors promotes proliferation, cell cycle progression and inhibits apoptosis indicating an oncogenic miR-221 function. Consequently, the tumor suppressor p27kip1, p57kip2, c-kit, Bim, ERα, PTEN, TIMP3, and PUMA have been reported to be miR-221 targets (17-20).

On the basis of our previous report we evaluated miR-221 as a prognostic marker in high-risk PCa in a larger patient cohort and an external validation. Furthermore we are the first to demonstrate a tumor suppressor function of miR-221 in PCa analyzing the mechanism by which this micro-RNA promotes tumor cell growth, invasiveness and apoptosis in PCa.

**Material and methods**

**Patients and samples:**

Consecutive men with high-risk PCa (PSA >20 ng/ml and/or clinical stage T3/4 and/or biopsy Gleason score 8-10), who had undergone radical prostatectomy (RP) between 1987 and 2005 at the Community Hospital of Karlsruhe, Germany (cohort 1) and the University Hospital Leuven, Belgium (cohort 2), were identified in the European Clinical and Translational High-Risk Prostate Cancer Research Group database (EMPaCT) and were included into this study. Clinical stage was assigned according to the 2002 TNM system, prostate biopsy cores were obtained under transrectal-ultrasound guidance, and pretreatment PSA was measured before digital rectal examination (DRE) or prostate ultrasound (US).

All patients were staged preoperatively with DRE, abdominopelvic computed tomography scan, and bone scan. Clinical node positive disease was not considered as exclusion criteria. None of the patients had received neo-adjuvant hormonal-, radiation- or chemotherapy. Prostate specimens were staged and graded according to the 2002 TNM classification and the Gleason grading system by two senior pathologists (P.S., E.L.). Follow-up was performed every 3 months for the first 2 years after surgery, every 6 months in the following 3 years, and
annually thereafter. Clinical failure (CF) was defined either as histologically proven local recurrence or distant metastasis confirmed by CT or bone-scan. Cause of death was verified by physician correspondence and/or death certificates and cancer related death (CRD) was defined as death due to prostate cancer. Overall survival (OS) was defined as time from RP to death of any cause, cancer specific survival (CSS) as the time from RP to death attributed to PCa or complications of the disease.

PCa-samples were paraffin-embedded tissue specimens from radical prostatectomy (regions with >90% cancerous tissue were used for the RNA extraction and qRT-PCR).

Clinical and pathological characteristics, CF-free survival and OS for both cohorts were comparable. After a median follow-up of 76 months (1-154) for cohort1 and 108 months (1-200) for cohort2 a total of 16 men (11.9%) and 15 men (16.9%) developed clinical failure and 11 (8.2%) and 12 (13.5%) of the men died PCa related, respectively. Also the estimated 10 and 15-years CSS rates were comparable for both patient groups (89% and 74% for cohort 1 and 87% and 78% for cohort 2). This study was approved by the local ethical committees (No. 59/04 and B322201214832 ). All included patients provided written, informed consent.

**RNA extraction of PCa samples and qRT-PCR:**

Total RNA for real-time PCR was extracted from the paraffin embedded PCa tissues with a Total RNA Extraction Kit (Applied Biosystems) as described previously (13). The RNA quality and concentration was determined with a BioAnalyzer (Agilent). cDNA was synthesized from total RNA with stem-loop reverse transcription primers for miR-221 according to the TaqMan MicroRNA Assay protocol. Mature miR expression was quantified in tissue samples with TaqManR microRNA assay kits and an Applied Biosystems 7900HT system according to the protocol provided in the manufacturer’s instructions (Applied Biosystems). The expression of miR-151-3p was used for normalization. Relative miR expression was calculated with the comparative ΔCt-method (ΔCt sample = Ct sample-Ct miR-151-3p);
ΔCt BPH = Ct BPH - Ct miR-151-3p). mRNA analysis of SOCS3 and IRF2 expression was performed according to standard qRT-PCR procedures. The expression of both GAPDH and β-Actin was used for normalization. All primer sequences are available under request. Mean Ct was always determined from triplicate PCRs.

**Cell cultures, generation of stable miR-221 overexpressing PC-3 clones, commercial growth assay and miRNA transfections:**

DU-145, PC-3 and LNCaP cells were purchased from the American Type Culture Collection (ATCC) and were grown in medium as indicated by ATCC instructions. Cells were transfected with human precursor miR-221 or negative control oligonucleotides using Lipofectamine following the manufacturer’s instructions (Applied Biosystems). The optimal miRNA oligonucleotide concentrations were titrated for optimal transfection results. In all experiments the final miRNA concentration was 10 nmol/L. To stably overexpress miR-221 we transfected PC-3 cells with a transposon vector and the pCMV(CAT)T7-SB100 expression plasmid for encoding the sleeping beauty transposase (21). Selection of the transgene was performed with puromycin (0.5μg/ml). The transposon vector was cloned by inserting the TurboRFP-miR-221 Fragment (Ecl136I/AfeI) of Tripz-miR221 into AfeI-cut pSB-ET (M.G. unpublished), which allows tetracycline regulated expression of the TurboRFP-miR-221 cassette. Puromycin resistant PC-3/miR-221 clones were picked and analysed for doxycycline (0.5μg/ml) induced expression of the TurboRFP-miR-221 cassette detecting RFP by fluorescence microscopy. MiR-221 overexpression was tested in doxycycline treated cells by qRT-PCR. Cell growth was analyzed by MTS assay (Promega) as indicated by the manufactures instructions as triplicates of 96 well cultures. Two days post transfection total RNA was extracted for RT-PCR and microarrays from cells cultured on 6 well plates using TRIzol reagent (Invitrogen) or PhosphoSave (Novagen) and used for expression analysis.
siRNA mediated knockdown of messenger RNA

Cells were grown in 96 well plates for MTS assays or in six well plates for total RNA and protein isolation. SiRNA transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufactures instructions. Cells were transfected with 5 nmol/L siRNA or control siRNA. Sequences for SOCS3 siRNA and IRF2 siRNA for targeting human SOCS3 or respectively human IRF2 were synthesized as published previously (22, 23). Control siRNA was purchased from Qiagen. DU-145 and PC-3 cells were cultured at a density of 4x10^5 cells/well and LNCaP cell were cultured at 8x 10^5 cells/well in six well plates. At day two post transfection cells on 6 well plates were harvested and total RNA or protein was isolated as described.

Microarray analysis:

Before labeling RNA quality was checked using a BioAnalyzer (Agilent). RNA integrity numbers (RIN) of the RNAs were 9.4 and 9.8. Total RNA was labeled according to Affymetrix standard protocols (IVT-express kit, Affymetrix Santa Clara, CA), without modification starting from 100ng and hybridized to a GeneChip® HG U 133 A 2.0 array. (Affymetrix). For the analysis of the resulting data different R packages from the Bioconductor project (www.bioconductor.org) were used. Signal intensities were normalized by variance stabilization normalization (vsn package, Bioconductor) and differential regulation of genes was assessed by a modified t-test (Limma package, Bioconductor) as described previously (13). A gene was regarded as being differentially expressed, if its log fold change > 1 and p-value < 0.05. For functional clustering, The Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/home.jsp) has been used. Additional functional clusters and text mining for gene interactions were generated through the use of IPA (Ingenuity Systems). The data discussed in this publication have been
deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE45627.

**Apoptosis assay**

Caspase-3/7 activity was analyzed using the Caspase-GLO 3/7 kit (Promega) as recommended by the manufacturer’s instructions. Cells were transfected with miRNAs or siRNAs in a 96 well plate as described. After 24 hours cells were incubated with medium supplemented with Caspase 3/7 reagent for 4 hours at room temperature. Cells were lysated and transferred to a white walled 96 plate for measurement of luminescence. Resulting data were expressed as OD values and normalized to untransfected control cells. Experiments were performed as triplicates.

**In vitro invasion assay**

A modified Boyden chamber assay was performed as described previously (24). PC-3 cells were cultivated in medium and transfected with pre miR-221 or pre miR-ctrl as described. After 48 h incubation and overnight starving in DMEM with 0.5% FCS, cells were seeded in the upper chamber of BSA-coated 8μM pore size transwell Boyden chambers (Corning star, Cambridge, MA, USA). Normal growth medium supplemented with 10% FCS was added to the bottom chamber as a chemo attractant and cells were allowed to migrate through the membrane for 6h. After removal of all cells remaining at the upper surface using a cotton carrier the lower surfaces of the membranes were stained for 30 s in a solution of 1% (w/v) crystal violet. Membranes were washed then with distilled water. Cell-associated crystal violet was extracted in 10% acetic acid and measured at 595 nm absorbance. The experiments were performed in triplicates.

**Western Blotting**
After harvesting, cells were washed twice by PBS and lysed in PhosphoSafe (Novagen) as recommended by the manufacturer’s instructions. Total protein concentrations were quantified (Bradford). Protein isolates were loaded on 12% SDS-PAGE gel with a concentration of 50 μg per lane resolved and transferred onto nitrocellulose membranes (BIO-RAD). The membranes were blocked using starting Block buffer (Invitrogen) and incubated at 4C with primary antibody following the manufacturers’ instructions. For protein expression by Western Blot we used following antibodies: SOCS3 (1:1000, ABCAM), IRF2, STAT1, pSTAT1, pSTAT3 and ERK 2 (all from Ambion) as loading control. We used horseradish peroxidase coupled secondary antibodies and the ECL Plus system (GE Healthcare) to visualize the protein expression and quantified band intensities using Image J program.

**Luciferase assays**

We used the Dual Luciferase Reporter assay system (Promega) as indicated by the manufactures instructions and analyzed the luciferase activity 48 hours after transfection. PCa cell lines were transiently transfected with pre-miR-221 as described. The 3’ untranslated regions of IRF2 and SOCS3 containing miR-221 binding sides were cloned into the pMIR-REPORT luciferase reporter vector (pMRL, Ambion). The contracts of the resulting pMRL-IRF2 or pMRL-SOCS3 vectors were cotransfected together with pre miR-221 or pre-miR ctrl. MiR-221 binding sites were identified by a bioinformatics search (Pictar, Targetscan, miRanda) and confirmed by alignment to the complementary miR-221 sequence. The 3’ UTR of human SOCS3 and IRF2 were amplified by PCR using following primers: SOCS3 Fw 5’-ACCAAGCTTGCCCACAGCCAGGAAGTG-3’ and SOCS3 Rw 5’-ACAACCTAGTCTGTCCAGGCCAATACCTG-3’; IRF-2 Fw 5’-TCACTAGTGTATTACATCCTTGTTGACCAC-3’ and IRF-2 Rw 5’-GAACTAGTGAAGTCTGAAAACGCCTCA-3’. Mutagenesis of miR-221 binding sides in the 3’ UTRs of IRF2 or SOCS3 were performed using the Site-Directed Mutagenesis Kit.
Primers for mutagenesis are as followed: SOCS3mut Fw 5’- GTGACAATTTCAGGAATCGATCAGCGATGGAATTACCTGGAACAG -3’ and SOCS3mut Rw 5’- CTGTTCCAGGTAATTCATCGCTGATCGATTCTGTAAATTGTAC-3’; IRF2mut Fw 5’-GGTGAAAAAAGCTTTTCGGCAACACTGTAGCAATCAGA-3’ and IRF2mut Rw 5’-TCTGATTGCTACATGAGTTGCCGAAAAGCTTTTTTAC-3’.

For all reporter assays, cells were transfected with 50 ng pMRL containing the mutated or wildtype SOCS3 3’UTR or IRF-2 3’ UTR and 50 nmol/L of pre miR-221 or pre miR-control.

**Statistical analysis of microRNA expression in the study cohort**

Relative miR-221 expression values display sample-specific characteristics. Based on the normalized miR-221 expression values, we determined Receiver Operating Characteristics (ROC) for various endpoints, precisely cancer related death (CRD) and clinical failure (CF). Endpoint-specific high/low miR-221 expression thresholds were determined based on ROC analysis such that cutoff values represent the optimal tradeoff between specificity and sensitivity. Survival was illustrated by Kaplan-Meier curves; survival differences between groups were examined with log-rank tests. The influence of miR-221 expression values as well as that of various clinical and epidemiological parameters was analyzed with univariate and multivariate Cox proportional hazard regression. The best fitting COX model was selected by measuring the relative goodness of fit with the Akaike information criterion (AIC). Differences in the mean values of miR-expression in two risk groups were analysed by two-sided Mann-Whitney test.

**Results:**

**MiR-221 as prognostic marker in high-risk PCa**

On the basis of our previous report indicating that miR-221 is a prognostic marker in PCa we analysed two independent high-risk PCa cohorts (cohort1 n=134; cohort2 n= 89) to validate
this finding. Patient selection and characteristics of both cohorts is provided in Supp. Fig. 1 and Supp. Tab. 1, respectively. In both cohorts we determined the miR-221 expression by RT-PCR and found downregulation in the large majority of the analysed PCa samples as compared to expression in BPH samples (data not shown). Reductions in mean miR-221 expression levels were identified between risk groups split by CRD but not for clinically used prognostic parameters indicating an association between progressive miR-221 downregulation and tumor aggressiveness in both cohorts (Fig.1A and Supp. Fig. 2). The prognostic value of miR-221 for predicting CRD in cohort 1 (learning cohort) was analyzed using receiver operating characteristics (ROC) analysis. The ROC analysis for CRD defined an optimal cutoff level ($\Delta$Ct, miR-221< -0.32) to dichotomize the patients into risk groups (Fig.1B). The calculated area under the curve (AUC) for CRD was 0.903 (Fig. 1B). Using this miR-221 cut off level we observed correct classification of 23.0% among the high risk cases (11 from 37) and respectively 100% among low risk cases (87 of 87). In Kaplan-Meier analysis low miR-221 expression ($\Delta$Ct, miR-221 <-0.32) was significantly correlated with CRD (Fig. 1C; P<0.0001). Cox proportional hazards regression analysis for time to CRD showed, that miR-221 expression, Gleason Score and lymph node invasion predicted CRD in univariate analysis. By stepwise regression analysis we generated a multivariate model for predicting CRD (determined by AIC), which contained miR-221, Gleason Score and lymph node invasion indicating that miR-221 functions as an independent predictor for CRD (Fig. 1D) in this model. The estimate of a hazard ratio (HR) for miR-221 was infinity since there were 100 % correct classification in one group.

To validate the predictive potential of the determined miR-221 cut off level we used cohort II (test cohort). Using the same cut-off level for miR-221 ($\Delta$Ct -0.32) as for cohort I we dichotomized the test cohort and performed Kaplan Meier estimates. Also in cohort II, low miR-221 expression correlated significantly with CRD (p<0.001) (Fig.1C). Among the high
risk group 10 of 20 (50.0%) and among the low risk group 68 of 69 (97%) cases were correctly classified by miR-221. Samples of the test cohort with miR-221 expression under the previously determined cut-off level were found to be associated with CRD by univariate Cox regression analysis (HR (95% CI) = 0.026 (0.003-0.201); p<0.0001)
As expected, miR-221 is also correlated with CF indicating that miR-221 can independently predict this outcome parameter either (Supp. Fig.3).

**Expression of miR-221 in prostate cancer cells causes growth inhibition, apoptosis and reduced invasive capabilities**

To analyze a tumor suppressor function of miR-221 we transiently transfected LNCaP, DU-145 and PC-3 cells with precursor-miR-221. We observed an efficient and strong miR-221 expression on day 2-post transfection by qRT-PCR in all three cell lines (Supp. Fig. 4 A). DU-145 and PC-3 cells responded to miR-221 re-expression by a significant decrease in cell proliferation (48% decrease in DU-145 and 69% in PC-3; p<0.01), while the androgen dependent LNCaP cells showed a moderate increase in proliferation (Fig. 2A). In concordance with the observed inhibition in proliferation in DU-145 and PC-3 cells, we found also reduced viability and changes in cell morphology after pre-miR-221 transfection (Fig. 2B). To prove that the decrease in cell viability is linked to induction of apoptosis we analyzed the activity of caspase3/caspase7. The caspase3/7-activity was significantly increased after miR-221 transfection in DU-145 and PC-3 cells, while LNCaP cells did not show increased apoptosis (Fig. 2C). We next assessed whether the expression of miR-221 had an impact on the invasive activities. Boyden chamber invasion assays showed reduced invasion levels in miR-221 transfected PC-3 cells (Fig. 2D). These results indicate that miR-221 acts as tumor suppressor in PC-3 and DU-145 PCa cells by regulating cell growth, apoptosis and invasiveness.
Global gene expression analysis of miR-221 re-expressing PC-3 cells

To search for molecular changes responsible for the observed biological effects we performed a microarray study on mRNA isolated from pre-miR-221 transfected PC-3 cells. This analysis revealed a set of significantly up- or downregulated genes in miR-221 re-expressing PC-3 cells (Fig 3A). We found that from 54,675 genes on the array 282 genes were up- and 64 downregulated (> 2-fold, p< 0.05) (Supp. Table 2). Many of the upregulated genes were known to be also upregulated by interferons. Validation of the array data using qRT-PCR assays confirmed this upregulation after miR-221 transfection for STAT-1, IRF1, IRF9, OSA1, IFI27 and IFI44 in PC-3 (Fig. 3B and Supp. Fig. 4B). Moreover we found downregulation of several potential oncogenes including PMEPA1 or PRUNE by qRT-PCR (Fig. 3C and Supp. Fig 4C), which might function as potential target genes for miR-221.

Pathway analysis revealed that miR-221 re-expression appeared to be preferentially associated with the TOLL-like receptor-, RIG-like receptor- or the JAK/STAT-pathways and that specifically interactions of the JAK/STAT pathway listed in the KEGG pathway showed changes (Supp. Fig. 5).

Since it was shown by several studies that transfection with synthetic small RNA molecules might randomly induce inflammatory cytokines like interferons (25, 26) we decided to generate PC-3 cells stable overexpressing miR-221. Using a transposon vector containing a TurboRFP-miR-221 fragment and the pCMV(CAT)T7-SB100 expression plasmid for encoding the sleeping beauty transposase we generated PC-3/miR-221 cell clones. Three out of three PC-3/miR-221 clones treated with doxycycline showed > 4 times overexpression of miR-221 (Supp. Fig. 6A). Analyzing two PC-3/miR-221 clones we confirmed miR-221 mediated growth inhibition and activation of interferon regulated genes (Supp. Fig. 6 B/C), confirming that miR-221 might specifically regulate the interferon signaling pathway in PC-3 cells.
MiR-221 expression induces STAT1 and STAT3 phosphorylation and sensitizes prostate cancer cells for the anti-proliferative effects of IFN-γ

To elucidate, if miR-221 expression is sufficient for STAT1 and STAT3 phosphorylation, we analyzed the expression of STAT1, pSTAT1 and pSTAT3 in pre-miR-221 transfected cells. MiR-221 re-expression activates STAT1 in PC-3 and DU-145 and induced STAT3 phosphorylation in DU-145 cell, but not in the STAT3 negative PC-3 cells (Fig. 4A).

It was previously shown that IFNs mediate their anti-proliferative function by phosphorylation and activation of the JAK/STAT pathway in PCa cells. In fact, we could observe that IFN-γ treatment of miR-221 transfected cells resulted in a significantly reduced proliferation (DU-145 cells 78%, PC-3 cells 81% reduction, p<0.01) compared to single IFN-γ treatment or untreated miR-221 transfected cells (Fig. 4B). In context with this IFN-γ sensitization we observed activation of STAT 1 and/or STAT3 in miR-221 re-expressing cells treated with IFN-γ (Fig 4A). Thus we observed additive effects in IFN mediated growth inhibition by miR-221 expression in PCa cells. In contrast, IFN-γ resistant LNCaP cells, that are known to be SOCS3 negative, did not show activation of STAT1 or inhibition of proliferation after IFN-γ treatment independent of miR-221 expression (Fig.4A).

MiR-221 targets IRF2 and SOCS3 and inhibits expression of IRF2 and SOCS3

Next we searched for miR-221 target genes, whose miR-221 mediated downregulation might be responsible for the observed biological effects. By in silico analysis we identified potential target sites in the 3’ UTR mRNA regions of IRF2 and SOCS3. Both genes are known negative regulators of the JAK/STAT signaling cascade. Therefore we analyzed the expression levels in miR-221 transfected cells and found moderate decrease in IRF2 or SOCS3 mRNA levels (Supp. Fig. 7) and a strong reduction in protein levels (Fig. 4A and Fig. 5B).
To demonstrate a direct interaction between miR-221 and IRF2 or SOCS3, we generated pGF-IRF2 and pGF-SOCS3 luciferase constructs, containing the miR-221 binding sites. In addition vectors with mutations at putative binding sites were cloned and used as controls. These vectors were co-transfected together with pre-miR-221 or scrambled miRNAs as negative controls followed by measurement of luciferase activity 48 h after transfection. As shown in Fig. 5A the luciferase activity in PC-3 cells co-transfected with constructs containing the 3’UTR of IRF2 or SOCS3 and pre-miR-221 was decreased by 62% (IRF2) and 41% (SOCS3).

**Downregulation of IRF2 or SOCS3 recapitulate the biological effects of miR-221 re-expression in prostate carcinoma cells**

To test whether IRF2 and SOCS3 are involved in the miR-221 mediated regulation of the JAK/STAT pathway we inhibited the expression of both genes in PC-3 cells. SiRNA knock down of both genes caused a strong and efficient decrease of protein levels (80-90%) (Fig. 5B). We also observed significantly reduced proliferation, induction of apoptosis and activation of STAT1 in response to siRNA mediated IRF2 or SOCS3 downregulation (Fig. 5C and D). We concluded that the biological effects caused by miR-221 overexpression are mediated at least partially by down-regulation of SOCS3 and IRF2.

**In vivo regulation of IRF-2 and SOCS-3 by miR-221 in prostate cancer**

To assess the role of miR-221 mediated inhibition of IRF2 and SOCS3 mRNA expression in primary PCa we selected a group of fresh frozen tumor samples on the basis of their miR-221 expression. In this series of PCa samples we correlated the expression of IRF2 and SOCS3 in response to miR-221 downregulation. As Fig. 6 shows we found an inverse correlation between miR-221 downregulation and upregulation of IRF2 or SOCS3 by Spearman rank correlation analysis whereas the mRNA expression of SOCS3 and IRF2 was not correlated.
We concluded that miR-221 is also in vivo critically involved in the expression of both potential target genes.

**Discussion:**

Based on the lack of prognostic models to accurately predict survival the need to better identify patients with lethal disease is one of the main challenges in PCa research. We previously demonstrated that miR-221 down-regulation hallmarks lymph node metastasis and possesses potential as a prognostic marker in high-risk PCa (13). Here we demonstrated that miR-221 predicted clinical failure and survival of high-risk PCa patients and determined a specific miR-221 expression level as independent predictive marker for CRD and CF. Using an independent test cohort we successfully validated the predictive power of miR-221 in predicting CRD and CF. The role of miR-221 as a prognostic biomarker is further supported by a recent report showing a correlation of miR-221 downregulation on BCR and CF in TMPRSS:ERG fusion positive PCa (27). The results presented here provide the groundwork to prospectively test miR-221 as a tissue based biomarker in high-risk PCa patients and to develop new treatment strategies within new clinical trial concepts (28).

Such new therapies are strongly related to the biological function of each individual microRNA. Several studies including our biomarker analysis clearly suggested a tumor suppressive function of miR-221 in PCa. Therefore, we analyzed the effects of re-expressing miR-221 in androgen independent PCa cell lines and demonstrated that miR-221 re-expression reduced proliferation, invasiveness and induced apoptotic cell death indicating a tumor suppressor role of miR-221 in androgen independent PCa cells.

To elucidate molecular pathways regulated by miR-221 we analyzed global mRNA expression profiles in miR-221 expressing PCa cells. Interestingly, besides the downregulation of oncogenic target genes like PMEPA1 and PRUNE (29, 30) we found an increased expression of genes, known to be associated with cell exposure to interferons.
Pathway analysis revealed activation of the Toll-like receptor, the RIG-like receptor and most impressively the JAK/STAT pathways. IFN mediated JAK/STAT activation in cancer development is not unexpected, because IFN usually functions as a cytokine with antitumor activity (31), moreover, there is a growing body of evidence that activation of the JAK/STAT pathway can inhibit proliferation and induce apoptosis in certain microenvironmental conditions. STAT1 is a known tumor suppressor involved in tumor development and expansion by switching on anti-proliferative and pro-apoptotic pathways (32, 33). We detected STAT1 and STAT3 phosphorylation in miR-221 re-expressing cells indicating a strong JAK/STAT pathway activation. An antitumorigenic activity by miR-221 mediated STAT3 activation is conflicting, because of its oncogenic function in some tumor entities (34-36). However, STAT3 activation can induce growth arrest and apoptosis under certain conditions in various cancer types including PCa (22, 37, 38). The activation of both STAT1 and STAT3 might explain at least partially the anti-proliferative and pro-apoptotic activity of miR-221 in PCa cells.

To elucidate how miR-221 expression activates the JAK/STAT pathway we identified SOCS3 and IRF2, both known negative regulator genes of the JAK/STAT pathway, as miR-221 targets. The role of SOCS3 as inhibitor of the JAK/STAT pathway in PCa is documented by the observation that STAT1 and STAT3 phosphorylation is inversely correlated with SOCS3 expression (39). Moreover, SOCS3 downregulation determined reduced proliferation rates and an increased apoptotic response by converting the anti-apoptotic STAT3 function into pro-apoptotic (40). It was also shown that reduced SOCS3 protein expression enhanced the IFN-γ responsiveness, indicating a regulation of IFN-γ sensitivity in PCa cells and other tumors by SOCS3 (22, 41).

In various cancer types IRF2 over-expression was found to be associated with the development and progression of malignant phenotypes. IRF2 acts as an antagonist to the tumor suppressor IRF1 and it is known that the IRF1/IRF2 balance is critically involved in the
immunomodulatory, antiproliferative, and pro-apoptotic IFN-γ effects (42, 43). Previous studies have shown that IRF1 and IRF2 are regulating transcription of the same IFN-γ–inducible genes (44), but with entirely opposing effects for cell growth and tumorigenicy (23). Thus, there is growing body of evidence that the IRF2 expression determines the cellular response to JAK/STAT pathway. Here we demonstrated that miR-221 re-expression induced IRF1 and down-regulated IRF2 expression. In addition we found that, similar as recently described for pancreatic cells, IRF2 knockdown leads to growth inhibition and apoptosis in PCa cells. Based on these results it is very likely that the downregulation of SOCS3 and IRF2 is responsible for the anti-tumorigenic biological effects in miR-221 re-expressing PCa cells. The relevance of these results for tumor development and tumor progression is further supported by the inverse correlation between miR-221 and SOCS3 or IRF2 expression in primary PCa probes. Fig. 7 summarizes a model how miR-221 regulates the JAK/STAT pathway and how miR-221 downregulation inhibits IFN-γ mediated antiproliferative and pro-apoptotic signals in PCa cells.

Our present study also provides evidence for a possible role of miR-221 to overcome the problem of low sensitivity against cytokine therapies in PCa. Interferon therapy was discussed for clinically advanced PCa (45). However, systemic IFN-γ therapy in PCa has shown only limited efficiency (46). We now demonstrate that miR-221 expression mediates the responsiveness against the antitumorigenic effects of IFN-γ in vitro. Moreover, we show evidence that miR-221 downregulation might reduce the IFN-γ responsiveness in primary PCa by upregulation of two independent negative regulator proteins (SOCS3 and IRF2) of this cytokine pathway. It is well known that the interaction of various negative regulatory proteins involved in cytokine signaling is very complex. Here we show that miR-221 is able to control such a signaling pathway by regulating various components and therefore it might be a good candidate for therapeutic use.
However, micro-RNA targeted therapy is challenging. Tissue specific delivery, stability, cellular uptake and off-target effects might be overcome by technical solutions in the future, but safety might remain a major concern in micro-RNA based therapy. Several cancer associated miRNA showed pivotal roles in tumor development and progression since a miRNA can function either as an oncogene if in a given cell type its critical target is a tumor suppressor or the same miRNA can be a tumor suppressor if in a different cell type its target is an oncogene (9). This seems to be true also for miR-221. Overexpression and regulation of tumor suppressor genes (i.e. p27kip1, Pten, etc.) were described for miR-221 in several tumor entities (14-17, 19, 20), while we and others showed that miR-221 is one of the most strongly and frequently down-regulated miRNA in primary PCa inhibiting the expression of the potential oncogenes IRF2 and SOCS3 (10-13). However, in PCa the situation seems to be even more complex, since miR-221 expression levels were shown to be increased in tumor tissue derived from bone metastasis of castration resistant prostate cancer (CRPC) (47, 48). Nevertheless, this observation is not per se mutually exclusive with the findings in the current study. While Sun et al. observed miR-221 overexpression only in CRPC we detected miR-221 downregulation in hormone naïve tumors. These observed differences implicate a specific function of miR-221 in the development of androgen resistance. Recent studies by Sun et al. supported this suggestion showing that the development of androgen independence in LNCaP cells was promoted via miR-221 mediated downregulation of HECTD2 and RAB1A re-programming the androgen signaling pathway (47). In contrast to these results we demonstrated that miR-221 overexpression activated the anti-proliferative and pro-apoptotic JAK/STAT pathway only in androgen-independent, SOCS3-positive DU-145 and PC-3, but not in androgen-dependent, SOCS3-negative LNCaP-cells. One possible explanation for the diverging results might be a pivotal function of miR-221 in the regulation of androgen independent growth and interferon signaling in presence or absence of SOCS3, since it was shown that sensitivity against androgen and interferon signaling in PCa cells depends on...
SOCS3 expression (49). Therefore we suggest that the different function of miR-221 in PCa cells at least partially depends on a SOCS3 mediated regulation of the androgen receptor- or the interferon- signaling pathway in PCa cells. Future \textit{in vitro} and \textit{in vivo} analysis describing a possible role of miR-221 in controlling various signaling pathway via posttranscriptional regulation of SOCS3 and other potential target genes might clarify this clinically relevant question.

In summary, we demonstrated for the first time that miR-221 has tumor suppressive function in PCa controlling apoptotic pathways, cell growth and invasiveness. The anti-tumorigenic effect of miR-221 expression is mediated at least partially by activation of the JAK/STAT pathway. We could show that miR-221 regulates two of the most important negative regulator proteins, SOCS3 and IRF2, of the JAK/STAT signaling pathway indicating a role of miR-221 as a master regulator of IFN-\(\gamma\) sensitivity in PCa cells. Moreover, we demonstrated that miR-221 expression is progressively decreased during PCa development and progression in clinical specimens and is an independent prognostic marker to predict cancer related death in high-risk PCa. On the basis of our results, we think that miR-221 has potential as a prognostic biomarker and as a target for future therapies of high-risk PCa.

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\textbf{References:}


Figures Legend:

Figure 1: miR-221 down-regulation predicts cancer related death in high-risk prostate cancer.

The analysis was performed in two independent patient cohorts originating from Germany (I) and Belgium (II). (A) Relative miR-221 expression levels (Δ\text{C}_t) of PCa samples of both cohorts were analyzed by qRT-PCR and subsequently divided into risk groups based on CRD. Significant reductions in the median expression levels were identified between the two groups in both cohorts and indicated by * \text{p} <0.001 using two-sided Mann-Whitney test. The cutoff level defined in the ROC analysis is indicated by the horizontal black line. (B) Receiver operating characteristic (ROC) analysis for predicting cancer related death (CRD) by miR-221 expression (Δ\text{C}_t). The prognostic value of miR-221 for prostate CRD was evident from areas under curve (AUC) of 0.9028 (learning cohort). An optimal ROC-derived threshold value to dichotomize the patients by normalized miR-221 expression was -0.325. (C) Kaplan-Meier analysis of patients with high-risk prostate cancer. Patients were grouped by the miR-221 expression cutoff defined in the ROC analysis; survival curves are shown for both groups. Low miR-221 expression is associated with earlier CRD (log-rank \text{p} <0.0001 in both groups). (D) Cox proportional hazards regression analysis for time to CRD revealed that dichotomised miR-221 expression level predicted cancer related death.

*) the actual HR is infinity, because one of the groups has no events (100% correct classification)
**Figure 2: Expression of miR-221 in PCa cells cause anti-tumorigenic effects**

A) MTS assay for the growth of indicated PCa cell lines that were transfected with pre miR-221 or pre-miR-precursor negative control (ctrl) and analyzed at day 2, 4 and 6 post transfection. Mock transfected cells showed no significant differences to ctrl cells and were excluded from the graph for better overview. Experiments were performed as triplicates. Data represent the mean +/- SD from five independent experiments. B) PC-3 cells were transfected with pre-miR-221, pre-miR precursor negative control (ctrl) or mock control. At day 6 post transfection pictures were captured (magnification 40x). C) Indicated PCa cell lines were transfected with pre miR-221 or pre miR precursor negative control (ctrl). Caspase3/7 activity was analyzed and was increased in PC-3 and DU145 cells transfected with pre-miR-221 when compared to control cells, but not in LNCaP cells. Results are presented in relation to the values measured in cells transfected with pre-miR precursor negative control that was arbitrary set as 1. Data represent mean values +/- SD of five independent experiments. (*, p<0.01 Wilcoxon rank sum test) D) miR-221 up regulation reduces cell migration of PCa cells. PC-3 cells were transfected with pre-miR-221 or control siRNA. Migration of PC-3 cells was measured over 6 h in a Transwell® cell culture chamber. Four chambers from three different experiments were analyzed (t-test; P<0.001). Each bar represents the mean ± SD. MiR-221 overexpression in pre miR-221 transfected PC-3 cells is shown in Supplement Fig.7.

**Figure 3: Comparison of mRNA expression patterns in PC-3 cells transfected with pre miR-221**

A) Heatplot of genes showing a log fold change > 2 and a p-value < 0.001 in a comparison of pre-miR-221 and pre miR precursor negative control transfected PC-3 samples. Pre miR-221 A and pre miR-221 B, respectively ctrl A and ctrl B, represent two independently performed experiments. B and C) For technical validation of array data we analyzed relative expression of selected genes, that were shown to be up- (B) or down-(C) regulated on the array.
Expression of indicated genes was significantly dysregulated in miR-221 transfected PC-3 cells. Normalized qRT-PCR results from miR-221 transfected cells are calculated as x-time expression changes in comparison to PC-3 cells transfected with pre miR-precursor negative control. Data represent mean values +/- SD of five independent experiments. The relative expression level of each gene in control transfected PC-3 cells was arbitrarily set as 1.0. Significant differences (P< 0.01) between expression in control and miR-221 transfected cells were indicated by asterisk (*). P values were calculated by student’s t-test.
Figure 4: MiR-221 expression induces STAT1 and STAT3 phosphorylation and sensitizes PCa cells for anti-proliferative effects of IFN-γ

A) PC-3, DU-145 and LNCaP cells were transfected with pre-miR-221 and pre-miR precursor negative control as indicated. On day one post transfection IFN-γ (10ng/ml) was added to the cell culture as indicated. At day two post transfection cells were harvested and Western Blots for STAT1 pSTAT1, STAT3, pSTAT3 SOCS-3 and Erk (loading control) were performed. Results show induction of pSTAT1 in PC-3 and DU-145 cells and induction of pSTAT3 in DU-145 cells after transfection with pre-miR-221 or by IFN-γ treatment. B) MTS assay for the growth of indicated PCa cell lines that were transfected with pre miR-221 or pre-miR precursor negative control (ctrl) in presence or absence of IFN-γ (10ng/ml). IFN-γ was added to the cell culture at day one post transfection. Cell cultures replicates were analyzed at day 2, 4 and 6 post transfection. Mock transfected cells showed no significant differences compared to ctrl cells and were not added to the graph for better overview. Experiments were performed as triplicates. Data represent the mean +/- SD from five independent experiments.

Figure 5: MiR-221 expression inhibits expression of IRF2 and SOCS3 and siRNA mediated downregulation of IRF2 and SOCS3 mimics effects of miR-221 re-expression in PCa cells

A) SOCS3 and IRF2 are targets of miR-221. SOCS3 and IRF2 luciferase constructs containing a wild-type or mutated SOCS-3 or IRF-2 3’ UTR, were cotransfected with pre-miR-221 in PC-3 cells. SOCS3 3’UTR or IRF2 3’UTR containing a mutation in the miR-221 binding side showed no significant difference in reporter activity compared to control transfected cells. Relative expression of firefly luciferase was standardized to control transfections. Luciferase activities were analyzed 48 hours after transfection. Reporter activities of cells cotransfected with miR-precur sor negative control (black bars) are arbitrary set as 1.0. The results were obtained from three independent experiments and are presented as
mean +/- SD. B) miR-221 re-expression or siRNA treatment decreased expression levels of SOCS3 or IRF2 and activated STAT1. PC-3 cells were transfected with negative control, pre miR-221 and SOCS3 siRNA or IRF2 siRNA for 48 hr. Western Blots were performed to analyze the expression of pSTAT1 and SOCS3 or pSTAT1 and IRF2. For both plots we used anti-ERK-2 as loading control. Western Blots were repeated at least three times showing comparable results. C) Effect of siRNA mediated knockdown of SOCS3 or IRF2 on the growth of PC-3 cells. MTS assay analysis for the growth of PC-3 cells. Cells were transfected with SOCS3 or IRF2 siRNA and control siRNA. On day 1 post transfection 10 ng/ml IFN-γ was added to the cultures when indicated. Cell cultures replicates were analyzed at day 2, 4 and 6 post transfection Experiments were performed in triplicates. Presented data are mean values +/- SD from three independent experiments. D) PC-3 cells (ctrl) were compared with IPC-3 cells transfected with pre-miR-ctrl, siRNA ctrl, pre miR-221, SOCS3 siRNA or IRF-2 siRNA. Caspase3/7 activity was analyzed 24 hours after transfection. Results are presented in relation to the values measured in not-transfected PC-3 cells, that was arbitrary set as 1. Data represent mean values +/- SD of three independent experiments. (*, p< 0.01 Wilcoxon rank sum test).

**Figure 6: Expression of miR-221 and SOCS3 or IRF2 is inversely regulated in human PCa**

A) Relative expression levels of miR-221, SOCS3 and IRF2 were analyzed by qRT-PCR in RNA extracts from fresh frozen human PCa samples and adjacent non-tumorigenic prostate tissue (n=30). Expression of miR-221, SOCS-3 and IRF-2 was calculated as x-fold overexpression in the cancer sample compared to the corresponding non-tumorigenic prostate sample. Subsequently the samples of the cohort were divided into subgroups based on a more than 2 fold downregulation (log fold change >-1 or <-1) of miR-221 and plotted against expression (calculated as log fold changes) of SOCS3 or IRF2 respectively. Results show
significant increased expression of SOCS3 and IRF2 in miR-221 downregulated PCa samples (black blots/miR-221<-1) when compared to PCa samples with no miR-221 downregulation (grey plots/miR-221>-1). B) plots showing the coefficient of correlation for relative expression levels of miR-221, SOCS3 and IRF-2 from the samples described above. CVs are shown in the blot.

Figure 7: Model of miR-221 tumor suppressor function in PCa.
We propose a model in which miR-221 downregulates SOCS3 and IRF2, which in turn, leads to an activation of the JAK-STAT pathway by STAT1 phosphorylation and increased IRF1 induced gene expression resulting in the activation of an apoptotic pathway, cell growth inhibition and decreased invasive activity. Modified based on: © 2000-2011 Ingenuity Systems, Inc. All rights reserved.
Fig. 1

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Fig. 7
Survival in high-risk prostate cancer patients is predicted by mir-221, which regulates proliferation, apoptosis and invasion of prostate cancer cells by inhibiting IRF2 and SOCS3

Burkhard Kneitz, Markus Krebs, Charis Kalogirou, et al.

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