Survival in Patients with High-Risk Prostate Cancer Is Predicted by miR-221, Which Regulates Proliferation, Apoptosis, and Invasion of Prostate Cancer Cells by Inhibiting IRF2 and SOCS3

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Introduction

In Europe, the number of newly diagnosed prostate cancer 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 period remained almost unchanged (75,000 in 1996 vs. 68,000 in 2006; refs. 1 and 2). The current inability to accurately distinguish risk of survival in patients with high-risk prostate cancer is one reason this disease is overtreated. miR-221 has been suggested as a biomarker in high-risk prostate cancer, 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 prostate cancer at least partially via STAT1/STAT3-mediated activation of the JAK/STAT signaling pathway. miR-221 directly inhibits the expression of SOCS3 and IRF2, two oncogenes that negatively regulate this signaling pathway. miR-221 expression sensitized prostate cancer cells for IFN-γ-mediated growth inhibition. Our findings suggest that miR-221 offers a novel prognostic biomarker and therapeutic target in high-risk prostate cancer.

Abstract

A lack of reliably informative biomarkers to distinguish indolent and lethal prostate cancer is one reason this disease is overtreated. miR-221 has been suggested as a biomarker in high-risk prostate cancer, 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 prostate cancer at least partially via STAT1/STAT3-mediated activation of the JAK/STAT signaling pathway. miR-221 directly inhibits the expression of SOCS3 and IRF2, two oncogenes that negatively regulate this signaling pathway. miR-221 expression sensitized prostate cancer cells for IFN-γ-mediated growth inhibition. Our findings suggest that miR-221 offers a novel prognostic biomarker and therapeutic target in high-risk prostate cancer.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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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 prostate cancer in a larger patient cohort and an external validation. Furthermore, we are the first to demonstrate a tumor suppressor function of miR-221 in prostate cancer analyzing the mechanism by which this microRNA promotes tumor cell growth, invasiveness, and apoptosis in prostate cancer.

Patients and Methods

Patients and samples

Consecutive men with high-risk prostate cancer [prostate-specific antigen (PSA) >20 ng/mL and/or clinical stage T3/4 and/or biopsy Gleason score 8–10], who had undergone radical prostatectomy 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.

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. Ströbel, E. Lerut). 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 was defined either as histologically proven local recurrence or distant metastasis confirmed by computed tomography or bone-scan. Cause of death was verified by physician correspondence and/or death certificates and CRD was defined as death because of prostate cancer. Overall survival (OS) was defined as time from radical prostatectomy to death of any cause, cancer-specific survival (CSS) as the time from radical prostatectomy to death attributed to prostate cancer or complications of the disease.

Prostate cancer samples were paraffin-embedded tissue specimens from radical prostatectomy (regions with >90% cancerous tissue were used for the RNA extraction and quantitative real-time PCR).

Clinical and pathologic characteristics, clinical failure–free survival, and OS for both cohorts were comparable. After a median follow-up of 76 months (1–154) for cohort 1 and 108 months (1–200) for cohort 2, 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 prostate cancer 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 B3220121/832). All included patients provided written, informed consent.

RNA extraction of prostate cancer samples and quantitative real-time PCR

Total RNA for real-time PCR was extracted from the paraffin-embedded prostate cancer 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 microRNA expression was quantified in tissue samples with TaqMan 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 sample / ΔCt miR-151-3p,BPH = Ct miR-151-3p,BPH – Ct miR-151-3p, BPH). mRNA analysis of SOCS3 and IRF2 expression was performed according to standard qRT-PCR procedures. The expression of both glycolaldehyde-3-phosphate dehydrogenase 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 (0.5 µg/mL). The transposon vector was cloned by inserting the TurboRFP-miR-221 cassette. Puromycin-resistant PC-3/miR-221 clones were picked and analyzed for doxycycline (0.5 µg/mL) induced expression of the TurboRFP-miR-221 cassette. Puromycin-resistant PC-3/miR-221 clones were picked and analyzed for doxycycline (0.5 µg/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 posttransfection, 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 6-well plates for total RNA and protein isolation. siRNA transfections
were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were transfected with 5 nM miRNA 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 fromQiagen. DU-145 and PC-3 cells were cultured at a density of $4 \times 10^5$ cells/well and LNCaP cell were cultured at $8 \times 10^5$ cells/well in 6-well plates. At day 2, posttransfection 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), without modification starting from 100 ng 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 (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 hours incubation and overnight starving in Dulbecco’s Modified Eagle Medium with 0.5% fetal calf serum, cells were seeded in the upper chamber of BSA-coated 8 μm/pore size Transwell Boyden chambers (Corning star). Normal growth medium supplemented with 10% fetal calf serum was added to the bottom chamber as a chemo attractant and cells were allowed to migrate through the membrane for 6 hours. After removal of all cells remaining at the upper surface using a cotton carrier the lower surfaces of the membranes were stained for 30 seconds 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 4°C with primary antibody following the manufacturers’ instructions. For protein expression by Western blot analysis, we used following antibodies: SOCS3 (1:1,000, 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. Prostate cancer cell lines were transiently transfected with pre-miR-221 as described. The 3’ untranslated regions of IRF2 and SOCS3 containing miR-221 binding sites were cloned into the pMIR-REPORT luciferase reporter vector (pMIR, Ambion). The constructs of the resulting pMIR-IRF2 or pMIR-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 Forward 5’-ACCAAGCTTCCACACGGAGGAAATG-3’ and SOCS3 Reverse 5’-AACAATCTTGTCACGCCCAATACCTG-3’; IRF2 Forward 5’-CTGCTACTCTTGTATTA-CATCCTTTTGTCC-3’ and IRF2 Reverse 5’-GAACAAGATCGATCATG-3’. Mutagenesis of miR-221 binding sites in the 3’ UTRs of IRF2 or SOCS3 was performed using the Site-Directed Mutagenesis Kit (QuickChange, Agilent Technologies). Primers for mutagenesis are as followed: SOCS3mut Forward 5’-GGATATTTCAGGGAGATCGATCAGGATGAT-3’ and SOCS3mut Reverse 5’-CTTCAAGTTTCTG-3’. Mutagenesis of miR-221 binding sites was performed using the Sites-Directed Mutagenesis Kit (QuickChange, Agilent Technologies). For all reporter assays, cells were transfected with 50 ng pMIR containing the mutated or wild-type SOCS3 3’ UTR or IRF2 3’ UTR and 50 nM of pre-miR-221 or pre-miR-control.

**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), without modification starting from 100 ng 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 (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.

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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 CRD and clinical failure. 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 2 risk groups were analyzed by the 2-sided Mann–Whitney test.

Results

miR-221 as prognostic marker in high-risk prostate cancer

On the basis of our previous report indicating that miR-221 is a prognostic marker in prostate cancer, we analyzed two independent high-risk prostate cancer cohorts (cohort 1, \( n = 134 \); cohort 2, \( n = 89 \)) to validate this finding. Patient selection and characteristics of both cohorts are provided in Supplementary Fig. S1 and Table S1, respectively. In both cohorts, we determined the miR-221 expression by RT-PCR and found downregulation in the large majority of the analyzed prostate cancer samples as compared with 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 Supplementary Fig. S2). The prognostic value of miR-221 for predicting CRD in cohort 1 (learning cohort) was analyzed using ROC analysis. The ROC analysis for CRD defined an optimal cutoff level (\( \Delta C_t \) 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 cutoff level, we observed correct classification of 23.0% among the high-risk cases (87 of 37) and respectively 100% among low-risk cases (87 of 87). In Kaplan–Meier analysis, low miR-221 expression (\( \Delta C_t \) 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 an HR for miR-221 was infinity because there were 100% correct classification in one group.

To validate the predictive potential of the determined miR-221 cutoff level, we used cohort 2 (test cohort). Using the same cutoff level for miR-221 (\( \Delta C_t \) −0.32) as for cohort 1, we dichotomized the test cohort and performed Kaplan–Meier estimates. Also in cohort 2, 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 cutoff 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 clinical failure, indicating that miR-221 can independently predict this outcome parameter either (Supplementary Fig. S3).

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 posttransfection by qRT-PCR in all three cell lines (Supplementary Fig. S4A). DU-145 and PC-3 cells responded to miR-221 reexpression by a significant decrease in cell proliferation (48% decrease in DU-145 and 69% in PC-3; \( P < 0.01 \)), whereas 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 also found 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 caspase-3/7. The caspase-3/7 activity was significantly increased after miR-221 transfection in DU-145 and PC-3 cells, whereas 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 prostate cancer cells by regulating cell growth, apoptosis, and invasiveness.

Global gene expression analysis of miR-221 reexpressing 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 reexpressing PC-3 cells (Fig. 3A). We found that from 54,675 genes on the array, 282 genes were upregulated and 64 downregulated (>2-fold; \( P < 0.05 \)); Supplementary Table S2). 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 Supplementary Fig. S4B). Moreover, we found downregulation of several potential oncopgenes.
including PMEPA1 or PRUNE by qRT-PCR (Fig. 3C and Supplementary Fig. S4C), which might function as potential target genes for miR-221.

Pathway analysis revealed that miR-221 reexpression seemed 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 (Supplementary Fig. S5). Because 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 of 3 PC-3/miR-221 clones treated with doxycycline showed >4 times overexpression of miR-221 (Supplementary Fig. S6A). Analyzing 2 PC-3/miR-221 clones we confirmed miR-221–mediated growth inhibition and activation of interferon-regulated genes (Supplementary Fig. 6B and C), confirming that miR-221 might specifically regulate the interferon-signaling pathway in PC-3 cells.

![Graph of miR-221 downregulation predicts cancer-related death in high-risk prostate cancer](image)

Figure 1. miR-221 downregulation predicts cancer-related death in high-risk prostate cancer. The analysis was performed in two independent patient cohorts originating from Germany (Group I) and Belgium (Group II). A, relative miR-221 expression levels (ΔCt) of prostate cancer 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 *; *P < 0.001 using 2-sided Mann–Whitney test. The cutoff level defined in the ROC analysis is indicated by the horizontal black line. B, ROC analysis for predicting CRD by miR-221 expression (ΔCt). The prognostic value of miR-221 for prostate CRD was evident from 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 P < 0.0001 in both groups). D, Cox proportional hazards regression analysis for time to CRD revealed that dichotomized miR-221 expression level predicted cancer-related death. *, the actual HR is infinity, because one of the groups had no events (100% correct classification).

### Table 1: Univariate and Multivariate Analysis of Prognostic Factors

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate HR (95% CI)</th>
<th>Univariate P</th>
<th>Multivariate HR (95% CI)</th>
<th>Multivariate P</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-221 (dichotomized)</td>
<td>*</td>
<td>*</td>
<td>P &lt; 0.0001</td>
<td>*</td>
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<tr>
<td>Gleason</td>
<td>1.97 (1.11–3.48)</td>
<td>P = 0.015</td>
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<td>PSA</td>
<td>1.08 (0.99–1.02)</td>
<td>P = 0.32</td>
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<tr>
<td>Lymph node invasion</td>
<td>0.44 (0.02–1.13)</td>
<td>P = 0.018</td>
<td>0.13 (0.016–0.99)</td>
<td>0.049</td>
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<tr>
<td>Age</td>
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<tr>
<td>pT</td>
<td>0.98 (0.29–3.22)</td>
<td>P = 0.97</td>
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miR-221 Is a Biomarker in Prostate Cancer and Inhibits IRF2 and SOCS3

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miR-221 expression induces STAT1 and STAT3 phosphorylation and sensitizes prostate cancer cells for the antiproliferative 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 reexpression 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 antiproliferative function by phosphorylation and activation of the JAK/STAT pathway in prostate cancer 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 with single IFN-γ treatment or untreated miR-221–transfected cells (Fig. 4B). In context with this IFN-γ sensitization, we observed activation of STAT1 and/or STAT3 in miR-221 reexpressing cells treated with IFN-γ (Fig. 4A). Thus, we observed additive effects in IFN-mediated growth inhibition by miR-221 expression in prostate cancer 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).

**Figure 2.** Expression of miR-221 in prostate cancer cells cause antitumorigenic effects. A, MTS assay for the growth of indicated prostate cancer cell lines that were transfected with pre-miR-221 or pre-miR-precursor negative control (ctrl) and analyzed at day 2, 4, and 6 posttransfection. Mock-transfected cells showed no significant differences to control cells and were excluded from the graph for better overview. Experiments were performed as triplicates. Data, 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, posttransfection pictures were captured (magnification, ×40). C, indicated prostate cancer cell lines were transfected with pre-miR-221 or pre-miR precursor negative control (ctrl). Caspase-3/7 activity was analyzed and was increased in PC-3 and DU145 cells transfected with pre-miR-221 when compared with 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 arbitrarily set as 1. Data, mean values ± SD of five independent experiments; *P < 0.01, Wilcoxon rank sum test. D, miR-221 upregulation reduces cell migration of prostate cancer cells. PC-3 cells were transfected with pre-miR-221 or control siRNA. Migration of PC-3 cells was measured more than 6 hours 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 as shown in Supplementary Fig. S7.
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 (Supplementary Fig. S7) and a strong reduction in protein levels (Figs. 4A and 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 cotransfected together with pre–miR-221 or scrambled miRNAs as negative controls followed by measurement of luciferase activity 48 hours after transfection. As shown in Fig. 5A, the luciferase activity in PC-3 cells cotransfected with pre–miR-221 and IRF2 or SOCS3 was decreased by 62% (IRF2) and 41% (SOCS3).

Downregulation of IRF2 or SOCS3 recapitulate the biological effects of miR-221 reexpression 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 knockdown 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 downregulation of SOCS3 and IRF2.

In vivo regulation of IRF2 and SOCS3 by miR-221 in prostate cancer

To assess the role of miR-221–mediated inhibition of IRF2 and SOCS3 mRNA expression in primary prostate cancer, we selected a group of fresh frozen tumor samples on the basis of their miR-221 expression. In this series of prostate cancer 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 prostate cancer research. We previously demonstrated that miR-221 downregulation hallmarks lymph node metastasis and possesses potential as a prognostic marker in high-risk prostate cancer (13). Here we demonstrated that miR-221 predicted clinical failure and survival of patients with high-risk prostate cancer and determined a specific miR-221 expression level as independent predictive marker for CRD and clinical failure. Using an independent test cohort we successfully validated the predictive power of miR-221 in predicting CRD and clinical failure. 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 clinical failure in TMPRSS:ERG fusion positive prostate cancer (27). The results presented here provide the groundwork to prospectively test miR-221 as a tissue-based biomarker in patients with high-risk prostate cancer 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 prostate cancer. Therefore, we analyzed the effects of reexpressing miR-221 in androgen independent prostate cancer cell lines and demonstrated that miR-221 reexpression reduced proliferation, invasiveness, and induced apoptotic cell death, indicating a tumor suppressor role of miR-221 in androgen independent prostate cancer cells.

To elucidate molecular pathways regulated by miR-221, we analyzed global mRNA expression profiles in miR-221 expressing prostate cancer cells. Interestingly, besides the downregulation of oncogenic target genes such as 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 antiproliferative and proapoptotic pathways (32, 33). We detected STAT1 and STAT3 phosphorylation in miR-221 reexpressing cells, indicating a strong JAK/STAT pathway activation. An antitumorogenic 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 prostate cancer (22, 37, 38). The activation of both STAT1 and STAT3 might explain at least partially the antiproliferative and proapoptotic activity of miR-221 in prostate cancer 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 prostate cancer 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 antiapoptotic STAT3 function into proapoptotic (40). It was also shown that reduced
SOCS3 protein expression enhanced the IFN-γ responsiveness, indicating a regulation of IFN-γ sensitivity in prostate cancer cells and other tumors by SOCS3 (22, 41).

In various cancer types, IRF2 overexpression 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 proapoptotic 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 tumorigenicity (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 reexpression induced IRF1 and downregulated IRF2 expression. In addition, we found that, similar as recently described for pancreatic cells, IRF2 knockdown leads to growth inhibition and apoptosis in prostate cancer cells. Based on these results it is very likely that the downregulation of SOCS3 and IRF2 is responsible for the antitumorigenic biological effects in miR-221 reexpressing prostate cancer 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 prostate cancer probes. Figure 7 summarizes a model how miR-221 regulates the JAK/STAT pathway and how miR-221 downregulation inhibits IFN-γ-mediated antiproliferative and proapoptotic signals in prostate cancer 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 prostate cancer. Interferon therapy was discussed for clinically advanced prostate cancer (45). However, systemic IFN-γ therapy in prostate cancer 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 prostate cancer 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, microRNA–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 microRNA–based therapy. Several cancer–associated miRNA showed pivotal roles in tumor development and progression because 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), whereas we and others showed that miR-221 is one of the most strongly and frequently downregulated miRNA in primary prostate cancer inhibiting the expression of the potential oncogenes IRF2 and SOCS3 (10–13).

Figure 7. Model of miR-221 tumor suppressor function in prostate cancer. 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.
However, in prostate cancer the situation seems to be even more complex, because miR-221 expression levels were shown to be increased in tumor tissue derived from bone metastasis of castration-resistant prostate cancer (CRPC; refs. 47 and 48). Nevertheless, this observation is not per se mutually exclusive with the findings in this study. Although Sun and colleagues 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 and colleagues supported this suggestion showing that the development of androgen independence in LNCaP cells was promoted via miR-221-mediated downregulation of HECTD2 and RAB1B re-programming the androgen signaling pathway (47). In contrast to these results we demonstrated that miR-221 overexpression activated the antiproliferative and proapoptotic 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 the presence or absence of SOCS3, because it was shown that sensitivity against androgen and interferon signaling in prostate cancer cells depends on SOCS3 expression (49). Therefore, we suggest that the different function of miR-221 in prostate cancer cells at least partially depends on a SOCS3-mediated regulation of the androgen receptor- or the interferon-signaling pathway in prostate cancer cells. Future in vitro and in vivo analysis describing a possible role of miR-221 in controlling various signaling pathways 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 prostate cancer controlling apoptotic pathways, cell growth, and invasiveness. The antitumorigenic 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-γ sensitivity in prostate cancer cells. Moreover, we demonstrated that miR-221 expression is progressively decreased during prostate cancer development and progression in clinical specimens and is an independent prognostic marker to predict cancer-related death in high-risk prostate cancer. 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 prostate cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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