MicroRNA-205, a novel regulator of the anti-apoptotic protein Bcl2, is downregulated in prostate cancer

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Abstract. Decreased expression of the microRNA miR-205 has been observed in multiple tumour types due to its role in the epithelial to mesenchymal transition, which promotes metastasis. We determined the expression of miR-205 in 111 archival samples of prostate carcinoma and found it to be strongly reduced in most samples, with a median expression level of 16% in comparison to benign tissue from the same patient. Lower miR-205 expression correlated significantly with tumour size and miR-205 levels decreased with increasing Gleason score from 7a=3+4 to 8=4+4. In addition, we describe the anti-apoptotic protein BCL2 as a target of miR-205, relevant for prostate cancer due to its role in prognosis of primary tumours and in the appearance of androgen independence. The repression of BCL2 by miR-205 was confirmed using reporter assays and western blotting. BCL2 mRNA expression in the same collective of prostate cancer tissue samples was associated with higher Gleason score and extracapsular extension of the tumour (pT3). Consistent with its anti-apoptotic target BCL2, miR-205 promoted apoptosis in prostate cancer cells in response to DNA damage by cisplatin and doxorubicin in the prostate cancer cell lines PC3 and LnCap. MiR-205 also inhibited proliferation in these cell lines.

Introduction

The expression of miR-205, transcribed from the MIR205HG gene on chromosome 1q, is specific for epithelial tissues (reviewed in ref. 1). Loss of expression of this microRNA in cancer has been found to be involved in the epithelial to mesenchymal transition (EMT), a reversible series of molecular changes that lead to the conversion of polarised immobile epithelial cells to more motile mesenchymal cells. EMT is required for embryonic development, but also occurs in epithelial tumours, leading to metastasis. Other microRNAs that are repressed during the EMT are the members of the miR-200 family, namely miR-200a, miR-200b, miR-200c, miR-141 and miR-429. The decreased expression of these miRNAs indirectly downregulates E-cadherin expression through the increased expression of ZEB1 and ZEB2, which repress the CDH1 gene (2), reviewed in (1). MiR-205 has been found to be downregulated in different tumour types, including breast cancer (3), head and neck cancer (4) and invasive bladder cancer, in contrast to non-invasive tumours (5). Microarray based screens of prostate cancer tissue also indicated a consistent downregulation of miR-205 (6-8). Nevertheless, upregulation of miR-205 in cancer has also been observed, e.g., in endometrial cancer (9,10), esophageal cancer (11) and non-small cell lung cancer (12). In the tumour types where increased expression of miR-205 occurs, it appears not to decrease cell growth, but it does still inhibit the EMT through repression of ZEB1 and ZEB2, which repress the CDH1 gene (2), reviewed in (1). MiR-205 has been found to be downregulated in different tumour types, including breast cancer (3), head and neck cancer (4) and invasive bladder cancer, in contrast to non-invasive tumours (5). Microarray based screens of prostate cancer tissue also indicated a consistent downregulation of miR-205 (6-8). Nevertheless, upregulation of miR-205 in cancer has also been observed, e.g., in endometrial cancer (9,10), esophageal cancer (11) and non-small cell lung cancer (12). In the tumour types where increased expression of miR-205 occurs, it appears not to decrease cell growth, but it does still inhibit the EMT through repression of ZEB2 (11) and inhibits metastasis (13).

Another confirmed target of this microRNA is the transcription factor E2F1 (14,15), which is involved in cell division and apoptosis. E2F1, in a negative feedback loop, appears to upregulate miR205 expression (16). In melanoma cell lines, miR-205 was also found to be positively regulated by full length p73 and repressed by the N-terminally truncated p73 isoform DNp73. Although p53 binds to the same sites as p73, it did not significantly regulate miR-205 in this system (16). In contrast, in breast cancer cell lines, a robust upregulation by p53 was seen. These authors did not test for the effects of p73, though (15). Positive regulation by DNp63, which also binds to the same sites at the promoter as p53 and p73, has also been described (17,18). In breast cancer it has recently been shown that overexpression of ErbB2 represses miR-205 leading to increased growth in the soft-agar assay (19), but whether this is a direct regulation remains elusive. Another possibility is silencing by methylation of the miR-205 promoter. This hypothesis is further supported by the fact that the CpG island located upstream of the first exon of miR205HG was found to be methylated in multiple prostate cancer cell lines (20) and
5’Aza treatment was able to increase miR-205 expression in breast cancer cell lines (15). In the present study, we investigated the downregulation of miR-205 in prostate cancer samples. The levels of miR-205 correlated significantly inversely with tumour size and decreased from Gleason score 7a=3+4 to 8=4+4. Additionally, we identified the anti-apoptotic gene BCL2 as a target of miR-205 in prostate cancer. We also correlated BCL2 expression to miR-205 levels in prostate cancer tissues. BCL2 is a potentially relevant miR-205 target specifically in prostate cancer, due to its known association to prognosis and disease progression (21,22).

Materials and methods

Patient collective. A series of 111 formalin-fixed paraffin-embedded (FFPE) prostatectomy specimens with primary prostate adenocarcinoma (PCa) from the Department of Pathology, Ruhr University Bochum, diagnosed between 2009 and 2011 were collected for the study. The composition of the cohort concerning Gleason grade, age, PSA levels and stage is shown in Table I. The PCa specimens were subjected to histological examination by an expert pathologist for confirmation of the Gleason grading, WHO classification of the tumour and staging according to the tumour-node-metastasis system. No patients had distant metastases at the time of diagnosis. The study was approved by the local Ethics Committee (Votum no. 3991-11).

Determination of miR-205 and BCL2 levels in patient samples. The histopathological tumour regions of interest were micro-dissected with the corresponding HE slide as a guide (tumour was marked with a pen on the HE slide) and used for RNA extraction using the miRNA FFPE kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions.

For the detection of miRNA levels, 500 ng total RNA was transcribed using the miScript reverse transcription kit (Qiagen); qRT-PCR was carried out with 25 ng cDNA per reaction using the miScript SYBR green PCR system (Qiagen) with the miR-205 primer (#MS00003780) or the RNU6B (U6 small nuclear RNA 2, #MS00014000) was used for the normalization to total RNA (23). For the detection of BCL2 in FFPE samples and cell culture, RNA was transcribed to cDNA using the High Capacity cDNA system (Life Technologies, Darmstadt, Germany). qRT-PCR was carried out with the TaqMan system (primer set #Hs99999901_s1) with 2X Universal Master Mix (Life Technologies). Template cDNA (25 ng) was used per PCR. 18S rRNA (#Hs99999901_s1) was used as internal control.

Cell culture. The prostate cancer cell lines PC3 and LnCap and the osteosarcoma cell line U2-OS were obtained from ATCC (Manassas, VA, USA). PC3 and LnCap cells were cultivated in RPMI-1640 (Pan Biotech, Aidenach, Germany) and U2-OS was cultivated in DMEM high glucose (Pan Biotech), both supplemented with 10% fetal calf serum (PAN Biotech); cisplatin and doxorubicin were obtained from Sigma-Aldrich (Taufkirchen, Germany).

Table I. Characteristics of the patient collective.

<table>
<thead>
<tr>
<th>Cohort feature</th>
<th>Median</th>
<th>Range</th>
<th>N</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>63.5</td>
<td>47.00-75.5</td>
<td>111</td>
</tr>
<tr>
<td>Tumour size (cm³)</td>
<td>2.30</td>
<td>0.16-18.00</td>
<td>111</td>
</tr>
<tr>
<td>PSA (ng/ml)</td>
<td>7.70</td>
<td>2.3-38</td>
<td>104</td>
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</table>

<table>
<thead>
<tr>
<th>Tumour characteristics</th>
<th>No. of samples (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Node-positive (N1) cases</td>
<td>6 (5.41)</td>
</tr>
<tr>
<td>Distant metastasis (M1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Lymphatic vessel invasion (L1)</td>
<td>8 (7.21)</td>
</tr>
<tr>
<td>Vascular invasion (V1)</td>
<td>1 (0.90)</td>
</tr>
<tr>
<td>Perineural invasion (Pn1)</td>
<td>65 (78.38)</td>
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<tr>
<td>Gleason sum</td>
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</tr>
<tr>
<td>3+3</td>
<td>14 (12.61)</td>
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<tr>
<td>4+3</td>
<td>47 (42.34)</td>
</tr>
<tr>
<td>4+4</td>
<td>37 (33.33)</td>
</tr>
<tr>
<td>3+4</td>
<td>13 (11.71)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>pT2a</td>
<td>10 (8.93)</td>
</tr>
<tr>
<td>pT2b</td>
<td>1 (0.89)</td>
</tr>
<tr>
<td>pT2c</td>
<td>72 (69.29)</td>
</tr>
<tr>
<td>pT3a</td>
<td>14 (12.50)</td>
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<tr>
<td>pT3b</td>
<td>13 (11.61)</td>
</tr>
<tr>
<td>pT4</td>
<td>1 (0.89)</td>
</tr>
</tbody>
</table>

Transfection of prostate cell lines with miRNA mimics. PC3 cells were seeded at 5x10⁵ per well and LnCap were seeded at 8x10⁵ cells per well in 6-well plates in their normal medium and transfected in suspension with 10 nM miRNA mimics (Qiagen) or AllStars negative control siRNA, labelled with AlexaFluor-647 (Qiagen), using 6 µl HiPerFect (Qiagen). The transfection efficiency was determined by measuring the fluorescence of the AllStars oligos by flow cytometry and was between 90 and 96%. Whenever the assay required, the cells were treated with cisplatin or doxorubicin 72 h after transfection.

Reporter assay. Fragments of the 3’ untranslated region (UTR) of E2F1 (262 bp), E2F5 (389 bp) and BCL2 (438 bp) containing the potential miR-205 binding sites were cloned into pGL3 as 3’UTR of the firefly luciferase gene by PCR. U2-OS cells were transiently transfected in triplicates with 40 ng of the pGL3 construct, 10 ng of the expression vector for renilla luciferase pRL-CMV (Promega, Mannheim, Germany) and 10 nmol of miRNA mimics (Qiagen), using the transfection reagent Attractene according to the manufacturer’s instructions (Qiagen). Cells were harvested 30 h after transfection and activities of firefly and renilla luciferase were measured using the Dual-Luciferase reporter assay system (Promega). The resulting luminescence was measured with a Tecan M200 microplate reader (Tecan, Crailsheim, Germany).
For statistical testing on expression levels of miR-205 and BCL2, data were log2 transformed, to allow the use of normal distribution-based tests and Pearson's correlation coefficient. For post hoc testing between individual pairs of conditions, the Scheffé test was applied. Statistical analysis was carried out by the Statistica 10 program (StatSoft, Tulsa, OK, USA).

Results

MiR-205 is downregulated in prostate cancer. We determined the expression of miR-205 by qRT-PCR in 111 FFPE specimens of prostate carcinoma. The miR-205 expression was found to be strongly reduced in 102 of 111 analyzed prostate cancer samples, with a median relative expression level of 0.160 in comparison to benign tissue from the same patient (interquartile range, IQR, 0.085-0.350). MiR-205 expression levels decreased with increasing Gleason score from 7a=3+4 to 8=4+4, but Gleason score 6=3+3 samples showed lower expression than Gleason score 7a samples (median expression levels: Gleason 3+6, 0.168; Gleason 7a, 0.303; Gleason 7b, 0.150; Gleason 8, 0.087; p=0.050 by ANOVA on log transformed data) (Fig. 1A). Its expression was also higher in tumours confined to the prostate (pT2) than in those that extend beyond the prostate (pT3) (p=0.0425). However, differences in miR-205 expression between perineural invasion categories were not significant (Fig. 1A). Only 9 of 111 samples showed an increased expression of miRNA 205 in tumour tissue in comparison to the benign controls; these were all relatively small tumours (<4 cm³). The inverse correlation
between miR-205 expression and tumour size was significant ($r=-0.234$, $p=0.0087$) (Fig. 1B).

The anti-apoptotic gene BCL2 is a target of miR-205. The reduced expression of miR-205 in prostate cancer indicates that its targets may aid tumour progression. From the targets suggested by the miRGen program (www.diana.pcbi.upenn.edu/miRGen/html), we selected BCL2 as a novel candidate. BCL2 appears interesting in this context, as its overexpression in prostate cancer is a known marker for poor prognosis (24).

In the ~5-kb long 3'UTR, 3 potential miR-205 binding sites are found, one weak (6-mer + a) centrally located at position 2380 and two potentially stronger sites near the 3' end of the 3'UTR at positions 3906 (7-mer-m8) and 4094 (7-mer-1a) (Fig. 2A), both with a $\Delta G$ above -13.40 kCal/mol (25). For a random sequence of this length, a $\Delta G$ of -13.40 kCal/mol would be expected (26). Additionally, these sites are located relatively close together and near the 3' end of a long 3'UTR, which are also typical characteristics of functional miRNA binding sites (27).

The fragment containing these two most 3' terminal sites was cloned into the pGL3 vector as 3'UTR for the luciferase gene (28). A reporter assay was done in U2-OS cells with miR-205 or control oligos to test for reduced luciferase activity after binding the microRNA. The osteosarcoma cell line U2-OS was used for this assay due to its ease of transfection and the stability of the assay in this cell line. As positive controls, equivalent constructs containing miR-205 binding sites in E2F1 and E2F5 were used. In comparison to the control oligos, miR-205 caused a 34% reduction in the luciferase signal when the BCL2 UTR was tested. In comparison, the E2F1 UTR caused a 51% percent reduction in signal intensity and the E2F5 UTR only 6%. The decrease in luminescence was significant for both BCL2 and E2F1 ($p<0.01$, t-test). The control vector did not show significantly reduced luciferase activity in the presence of miR-205 (Fig. 2B).

Next, we analyzed if overexpression of miR-205 leads to a decrease in the BCL2 protein levels of the prostate cancer cell line PC3. We observed a decrease in the BCL2 level by western blotting at 72 and 96 h after transfection with miR-205 mimics, in comparison those transfected with control oligos (Fig. 2C).

BCL2 expression in comparison to miR-205 levels in patient samples. Having confirmed BCL2 as a miR-205 target, we determined the expression of BCL2 at the mRNA level by TaqMan assay in the series of archival samples for which miR-205 levels were measured by qRT-PCR. The quality of the RNA was sufficient for reliable PCR results in 84 of 111 samples. The mRNA levels of BCL2 have been found to correlate well with its protein level (29), allowing for the use of qRT-PCR to measure BCL2 expression. BCL2 is strongly expressed in the basal cells of benign glands (24,30), making the comparison to benign tissue difficult for this
gene. Therefore, we calculated BCL2 expression levels as ∆Ct relative to 18S levels in tumour tissue. BCL2 mRNA expression increased significantly with increasing Gleason grade (median expression relative to 18S: Gleason 3+3, 0.0074% (n=12); Gleason 3+4, 0.0148% (n=33); Gleason 4+3, 0.022% (n=31); Gleason 4+4, 0.100% (n=8); p=0.173. Expression was also stronger in tumours with extension beyond the prostate (pT3, median, 0.0488%) in comparison to those confined to the prostate (pT2, median, 0.0148%) (p=0.0399). There was no significant difference between those with (Pn1, median, 0.0172%) and without perineural invasion (Pn0, median, 0.0118%) (Fig. 3A). BCL2 levels did not correlate to tumour size either.

Although there was no linear correlation between miR-205 and BCL2 expression, all the samples except one with high BCL2 expression, had low (<30% of benign) miR-205 expression (Fig. 3B).

**MiR-205 promotes apoptosis in prostate cancer cells.** Having confirmed anti-apoptotic gene BCL2 as a miR-205 target, we investigated the effect of miR-205 overexpression on the induction of cell death in prostate cancer cells. We measured caspase 3/7 activity in cells transfected with miR-205 mimics after treatment for 24 h with 0.1 µg/ml doxorubicin (0.1724 µM), 4 and 10 µM cisplatin (PC3) or 10 and 20 µM (LnCap) cisplatin. A consistent increase in caspase activity was observed in cells transfected with miR-205 in comparison to control oligos in both cell lines in combination with cisplatin treatment (Fig. 4A). Cisplatin (4 µM) does not induce significant cell death in LnCap cells, therefore higher doses were used in this cell line (Fig. 4B and data not shown). Doxorubicin only caused a significant difference in PC3 cells in this assay.

As a marker for apoptosis, we determined the sub-G1 fraction by flow cytometry 48 h after genotoxic treatment. We observed a significant increase in the fraction of apoptotic cells both in control cells and cells treated with doxorubicin or 4 µM cisplatin (Fig. 4C) in PC3. LnCap shows the strongest increase in apoptosis at 10 µM cisplatin concentration in combination with miRNA205 mimics. In these cells there was no increase in the spontaneous apoptosis rate after miR-205 transfection, in contrast to PC3. Apoptosis is also characterized by loss of mitochondrial membrane potential (∆ΨM), which can be measured through a change in the fluorescence spectrum of the JC-1 dye. Loss of ∆ΨM was significantly increased in LnCap cells transfected with miR-205 after treatment with 10 µM cisplatin, as compared to control transfected cells, confirming the results for caspase 3/7 activation (Fig. 4B).

**MiR-205 inhibits cell division in prostate cancer cells.** We next determined the effect of miR-205 overexpression on cell growth. For this, PC3 and LnCap cells were transfected with either AllStars negative control siRNA or miR-205 mimics and then plated out at low densities. An MTT assay was performed at the starting point and at 3, 5 and 7 days thereafter. We observed a significantly lower cell number in the miR-205 transfected cells (p<0.05), in comparison to control transfected cells at 7 days (Fig. 5) in both cell lines.

**Discussion**

In this study, we describe the downregulation of the microRNA miR-205 in prostate cancer, which is known to be involved in the epithelial to mesenchymal transition (1,20). In our series of samples, expression of miR-205 was reduced in 76 of 84 prostate cancer specimens, with a median relative expression level of 18.0% in tumour tissue compared to benign tissue of the same patient. This finding is in accordance with earlier data from microarray experiments (7,8) and from qRT-PCR in a smaller series of patients (31). The level of miR-205 correlated inversely with tumour size, which fits with the growth inhibitory effects we observed for this microRNA. Moreover, there was a trend for miR-205 expression to decrease with increasing Gleason grade from 7a (3+4) to 8 (4+4). This is noteworthy as it corresponds to the worse prognosis of patients with primarily Gleason grade 4 as opposed to primarily Gleason grade 3 (32).

The role of miR-205 in cancer development depends on the tissue type, as it has been found to be either overexpressed or downregulated, depending on the tumour origin. Its functions include the prevention of epithelial to mesenchymal transition, which is associated with metastasis, through repression of ZEB1 and ZEB2 and consequent high E-cadherin levels (reviewed in ref. 1). On the other hand, several of its described targets, such as E2F1 (14), PTEN (33),
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Figure 4. MiR-205 increases apoptosis after DNA damage in PC3 and LnCap cells. (A) Induction of caspase 3/7 activity in PC3 (left panel) and LnCap (right panel) cells transfected with miR-205 mimics or AllStars control oligos after treatment with DNA damaging agents. Cells were treated for 24 h for PC3 and 48 h for LnCap before measurement. (B) Loss of mitochondrial membrane potential upon cisplatin treatment is enhanced after overexpression of miR-205 in PC3 cells (left panel) and LnCap cells (right panel). (C) Increase in sub-G1 fraction in PC3 cells (left panel) and LnCap cells (right panel) transfected with miR-205 or control oligos were treated for 48 h with doxorubicin or cisplatin at the indicated concentrations. There was a consistent increase in the sub-G1 fraction after miR-205 transfection. *Significant difference with p<0.05; **p<0.01; ***p<0.001.

Figure 5. MiR-205 reduces cell growth in PC3 and LnCap cells. PC3 (A) and LnCap (B) cells were transfected with miR-205 or control RNA oligos. A MTT assay was done 1, 3, 5 and 7 days post-transfection. The differences between the cell number at day seven were significant for both cell lines (LnCap, **p=0.0065; and PC3, *p=0.0486).
miR-205

In the present study, we describe the anti-apoptotic protein BCL2 as a novel target of miR-205. This protein is likely an important target in prostate cancer, as high BCL2 expression in primary prostate cancer is a marker for poor prognosis, with an increased risk for recurrence (21,22,24,34). Biochemical recurrence during anti-androgen therapy is frequently associated with increased BCL2 expression (35,36). Loss of miR-205 may play a role in androgen independence, as one study reported reduced expression only in androgen-independent tumours (7), which would then correspond to higher BCL2 levels.

The regulation of BCL2 expression in prostate cancer is complex. It is a known target of several other microRNAs, such as miR-15, miR-16 (37) and the miR-34-family (38,39). A different and independent mechanism for low BCL2 expression in prostate cancer is methylation of the BCL2 promoter (30,40), which would explain why many cases do not express BCL2 at all, whereas those that express it, are strongly positive. Gene rearrangements of BCL2 appear to be rare in prostate cancer (21). Taken together, these factors may explain the poor correlation between BCL2 levels in vivo in our samples, in contrast to the in vitro results in PC3 and LnCap cells. Further research will be needed to clarify this issue.

With BCL2 as a target, in combination with the expected effects of previously published targets like E2F1, we found that overexpression of miR-205 in prostate cancer cell lines decreases cell division and increases apoptosis in response to chemotherapeutical agents. Moreover, these data are in accordance with results in different prostate cancer cell lines after treatment with cisplatin and docetaxel. The authors attribute their results to repression of BCL-W (encoded by BCL2L2), which is another anti-apoptotic member of the BH3 family (20). As we confirmed the repression of the anti-apoptotic BCL2 by miR-205, the observed effects may also in part be caused by this protein.

In conclusion, we identified the downregulation of miR-205 in prostate cancer and describe BCL2 as a novel target of this microRNA, with potential prognostic and therapeutic implications. Increased expression of miR-205 was found to inhibit cell growth and to sensitize cells to apoptosis, caused by chemotherapeutical agents in a BCL2-dependent manner.

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References

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