Identification of a hypoxia-regulated miRNA signature in bladder cancer and a role for miR-145 in hypoxia-dependent apoptosis

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Background: Hypoxia leads to the stabilisation of the hypoxia-inducible factor (HIF) transcription factor that drives the expression of target genes including microRNAs (miRNAs). MicroRNAs are known to regulate many genes involved in tumourigenesis. The aim of this study was to identify hypoxia-regulated miRNAs (HRMs) in bladder cancer and investigate their functional significance.

Methods: Bladder cancer cell lines were exposed to normoxic and hypoxic conditions and interrogated for the expression of 384 miRNAs by qPCR. Functional studies were carried out using siRNA-mediated gene knockdown and chromatin immunoprecipitations. Apoptosis was quantified by annexin V staining and flow cytometry.

Results: The HRM signature for NMI bladder cancer lines includes miR-210, miR-193b, miR-145, miR-125-3p, miR-708 and miR-517a. The most hypoxia-upregulated miRNA was miR-145. The miR-145 was a direct target of HIF-1α and two hypoxia response elements were identified within the promoter region of the gene. Finally, the hypoxic upregulation of miR-145 contributed to increased apoptosis in RT4 cells.

Conclusions: We have demonstrated the hypoxic regulation of a number of miRNAs in bladder cancer. We have shown that miR-145 is a novel, robust and direct HIF target gene that in turn leads to increased cell death in NMI bladder cancer cell lines.
Hypoxia-regulated miRNAs and miR-145

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Figure 1. Hypoxia-regulated miRNAs (HRMs) in bladder cancer. (A) Venn diagram representation of the 30 most induced miRNAs in RT4 and RT112 cells. (B) Median fold change from three independent experiments of the 10 most induced miRNAs in RT4 and RT112 cells.

1. miR-145 - 68.7
2. miR-210 - 15.4
3. miR-518-3p - 7.9
4. miR-125a-3p - 5.7
5. miR-519d - 5.6
6. miR-708 - 4.7
7. miR-525-3p - 3.5
8. miR-517a - 2.3
9. miR-519a - 2.9
10. miR-335 - 2.4

1. miR-518f - 36.3
2. miR-515 - 15.0
3. miR-210 - 5.9
4. miR-200a - 5.2
5. miR-15a - 5.0
6. miR-99a - 4.6
7. miR-107 - 4.4
8. miR-106b - 3.4
9. miR-146-5p - 2.6
10. miR-212 - 2.4

1. miR-100 - decreased

www.bjcancer.com | DOI:10.1038/bjc.2015.203

635
lines (Blick et al., 2013). However, to date, no comprehensive analysis has been performed to identify HRMs in bladder cancer. The initial aim of this study was to identify HRM signatures in NMI and MI bladder cancer. Furthermore, we go on to demonstrate that one of the miRNA in the signature, miR-145, is a bona fide HIF target in NMI bladder cancer and show that it plays a role in controlling cell viability after sustained exposure to hypoxia.

MATERIALS AND METHODS

Cell culture. The cell lines RT4, RT112, T24 and HT1376 were obtained from Cancer Research UK Cell Services (Clare Hall Laboratories, London, UK) and cultured as previously described (Blick et al., 2013). A hypoxia incubator (MiniGalaxy A, RS Biotech, Irvine, Scotland) or hypoxia workstation (In Vivo2, Ruskim Technology, Bridgend, UK) were used to achieve low oxygen conditions (1% or 0.1% O2 respectively) in parallel to cells maintained in normoxic conditions (5% CO2, 37°C, 21% O2) for the indicated time. All experiments were done in triplicate from independent cell cultures.

RNA extraction, reverse transcription and quantitative PCR (qPCR) for microRNAs. Cells were lysed with Tri Reagent (Sigma-Aldrich, St Louis, MO, USA), and RNA extracted using chloroform followed by ethanol precipitation. RNA (350 ng) was reverse transcribed using the TaqMan Megaplex Primer Pool A

Figure 2. Validation of common HRMs in bladder cancer cell lines. Expression of (A–D) miR-210 and (E–H) miR-193b in (A and E) RT4, (B and F) T24, (C and G) RT112 and (D and H) HT1376 cells exposed to normoxia (N; white bars), 1% O2 (hatched bars) or 0.1% O2 (black bars) for the indicated time. Data are mean and s.e.m. of three independent experiments. *P<0.05, **P<0.01, ***P<0.001.
mix and the TaqMan Reverse Transcription Kit (Applied Biosystems, Warrington, UK). The miRNA expression was assayed on either the TaqMan Array Human MicroRNA A Card or using individual assays (Applied Biosystems). Real-time PCR runs were done on the 7900HT Fast Real Time PCR System (Applied Biosystems). For each miRNA, each sample was assayed in triplicate. The miRNA expression was normalised using either RNU44 or RNU48 using the comparative Ct method (Livak and Schmittgen, 2001) and presented as fold change relative to expression in normoxia.

**The siRNA, miRNA mimics and anti-miRs.** The siRNAs against HIF-1α and HIF-2α have been previously described (Blick et al., 2013). The scramble (Scr) and siRNA sequence against p53 were synthesised by Eurogentec (Liege, Belgium) and are as follows: Scr: 5'-ACGACACGCAGGUGGUCAU-3' and sip53: 5'-GACUC CAGUGUAUCUAC-3'. The miR-145 mimic (mimic-miR-145) and anti-miR (anti-miR-145) and appropriate controls were purchased from Dharmacon (Lafayette, CO, USA).

**Transfection protocol.** Cells were reverse transfected with siRNA, mimic-miR-145 or anti-miR-145 with Oligofectamine (Invitrogen, Paisley, UK) according to the manufacturer’s instructions as previously described (Blick et al., 2013).

**Chromatin immunoprecipitation.** Chromatin immunoprecipitations (ChiPs) were performed using the EZ-ChIP kit (Millipore, Billerica, MA, USA).

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**Figure 3. Validation of HRMs in RT4 cells.** Expression of (A) miR-518-3p, (B) miR-125a-3p, (C) miR-519-19d, (D) miR-708, (E) miR-525-3p, (F) miR-517, (G) miR-519a and (H) miR-335 in RT4 cells exposed to normoxia (N; white bars), 1% O2 (hatched bars) or 0.1% O2 (black bars) for 24 h. Data are mean and s.e.m. of three independent experiments. *P<0.05, **P<0.01, ***P<0.001.
Watford, UK) according to the manufacturer’s instructions. Briefly, RT4 cells were either incubated in normoxia or 0.1% O2 for 24 h. Proteins were first crosslinked to DNA with 1% formaldehyde for 10 min at room temperature and excess formaldehyde was quenched with 125 mM glycine for 5 min. Cells were harvested by scraping and resuspended in SDS lysis buffer with protease inhibitors to get a cell suspension at 2 × 10^7 cells per ml. Genomic DNA was sheared using a Diagenode Bioruptor (Liege, Belgium) sonicator to get fragments sizes in the range of 250–750 bp. Sheared DNA was precleared with protein G beads and immunoprecipitations were performed with anti-HIF-1α and anti-RNA polymerase II antibodies using 100 μl of sheared DNA per antibody. After overnight incubation at 4 °C, antibodies with bound chromatin were purified with protein G beads, the protein/DNA complexes eluted and then the crosslinks reversed to release the sheared genomic DNA.

**Primer**s. Primers were purchased from Invitrogen. The following primer pairs were used for the ChIP PCRs: miR-145 HRE1_F: 5'-GTGAATGAGGCCGTGAACAGAGAC-3' and miR-145 HRE1_R: 5'-CATGTCCAAGGTTCTAGTTCCTTGG-3'; miR-145_HRE2_F: 5'-AGCACGGGCGAGGTCAG-3' and miR-145_HRE2_R: 5'-GGCAAATTGATGACACTG-3'; CA9_HRE_F: 5'-GTCCATGGCACCATAAAGCTCTTGGGGGG-3' and CA9_HRE_R: 5'-GGGCTCCTAACCTGTTCGATGGAC-3'; UBC_F: 5'-TTGCTGGCAATATCGGACG-3' and UBC_R: 5'-GCAAGACCATCACCCTTGAG-3'.

Flow cytometry. RT4 cells were reverse transfected with mimic-145 or anti-miR-145 and appropriate controls and either kept in

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**Figure 4. Validation of HRMs in T24 cells.** Expression of (A) miR-518f, (B) miR-200a, (C) miR-99a, (D) miR-107, (E) miR-194, (F) miR-212, (G) miR-15a and (H) miR-150 in T24 cells exposed to normoxia (N; white bars), 1% O2 (hatched bars) or 0.1% O2 (black bars) for 24 h. Data are mean and s.e.m. of three independent experiments. *P<0.05, **P<0.01, ***P<0.001.
normoxia or placed in 0.1% O_2. On days 3, 4 and 5, cells were harvested with trypsin and pelleted. Cells were stained with annexin-V-AlexaFluor 647 (Invitrogen; 5 μl) and propidium iodide (Invitrogen; 50 μg/ml) in 100 μl of annexin V binding buffer (BD Pharmagen, San Jose, CA, USA) for 15 min at room temperature. Subsequently, 400 μl of annexin V staining buffer was added to each sample, cells were placed on ice and analysed by flow cytometry using a Cyan ADP Flow Cytometer (Beckman Coulter, Indianapolis, IN, USA). Data collection and analysis was performed using Summit version 1 (DAKO, Glostrop, Denmark).

**Correlation of miRNA expression.** We have previously characterised the expression of miR-210, miR-193b and miR-145 in 55 primary bladder cancer samples (both NMI and MI) and normal urothelium (n = 20) by qPCR (Catto et al, 2009). The correlation of normalised expression of miR-145, miR-210 and miR-193b was investigated by linear regression.

### RESULTS

Hypoxia regulates both common and distinct miRNAs in MI and NMI bladder cancer. To generate HRM signatures in bladder cancer, the expression of 384 miRNAs in the TaqMan miRNA pool A were analysed in the noninvasive cell line RT4 and the invasive cancer, the expression of 384 miRNAs in the TaqMan miRNA pool and NMI bladder cancer.

Hypoxia regulates both common and distinct miRNAs in MI and NMI bladder cancer. We investigated the expression of miR-145 (Figure 5), significant hypoxic upregulation of miR-145 in both cell lines (Supplementary Figure 1). The most robust HRM miR-210 was induced after exposure to low oxygen in all bladder cancer cell lines (Figure 2C). In addition, miR-193b was also induced by hypoxia in all cell lines except T24 (Figure 2E). In agreement with the expression of these two miRNAs in h-TERT, an immortalised normal urothelial cell line. Both miR-210 and miR-193b were upregulated in response to hypoxia in h-TERT cells, although the fold induction was lower than those observed in the bladder cancer cell lines (Supplementary Figure 2).

In addition to miR-145 (Figure 5), significant hypoxic upregulation of miR-18-3p, 125a-3p, 517a, 519a and 335 was confirmed in RT4 cells (Figure 1A). MiR-525-3p and miR-519d were not significantly induced in response to hypoxia in RT4 cells (Figure 1B). MiR-518-3p, 125a-3p, 519a and 335 were confirmed in RT4 cells (Figure 1A). MiR-525-3p and miR-519d were not significantly induced in response to hypoxia in RT4 cells (Figure 1B). MiR-518-3p, 125a-3p, 519a and 335 were confirmed in RT4 cells (Figure 1A). MiR-525-3p and miR-519d were not significantly induced in response to hypoxia in RT4 cells (Figure 1B).

**Figure 5. Regulation of miR-145 by hypoxia and p53.** (A) RT4 cells were cultured in normoxia (white bars), 1% O_2 (hatched bars) or 0.1% O_2 (black bars) for 24 h. (B) RT4 cells were cultured in normoxia (N; white bars), 0.1% O_2 (black bars) or in normoxia and treated with DMOG (hatched bars) for 24 h. (C) Expression of miR-145 and (D) expression of miR-210 in RT4 cells cultured in normoxia (white bars) or 0.1% O_2 (black bars) for 24 h after transfection with scramble (Scr) siRNA or siRNA against HIF-1α or HIF-2α. Data are mean and s.e.m. of three independent experiments. *P<0.05, **P<0.01.

In RT4, miR-145 was the most robust HRM, being induced more strongly than miR-210 (Supplementary Figure 1 and Figure 1B). Furthermore, a number of miRNAs belonging to the C19MC cluster on chromosome 19 (Bortolin-Cavaille et al, 2009; Ren et al, 2009) including miR-518a-3p, 515-5p and 525-3p were also upregulated in response to hypoxia in this line. In T24, three members of the miR-200 family, including miR-200a, 200b and 200c, were upregulated in response to hypoxia (Supplementary Figure 1). Additional miRNAs upregulated in response to hypoxia in T24 cells included miR-150 and miR-15a. Across both cell lines, only 3 miRNAs were upregulated >10-fold in hypoxia: miR-145 and miR-210 in RT4 and miR-518f in T24 (Figure 1B). Validation of low-density arrays reveals novel HRMs in bladder cancer cell lines. To validate the findings of the arrays, the expression of the following miRNAs was examined with individual assays – miRs 145, 518-3p, 125a-3p, 519d, 708, 525-3p, 517a, 519a, and miR-335 in RT4 and miRs 518f, 150, 200a, 15a, 99a, 107, 194 and 212 in T24. Validated targets in RT4 were also examined in a second NMI line, RT112, whereas confirmed targets in T24 were investigated in another MI line HT1376. The expression of miR-210 and miR-193b were examined in all cell lines. The robust HRM miR-210 was induced after exposure to low oxygen in all bladder cancer cell lines (Figure 2A–D). In addition, miR-193b was also induced by hypoxia in all cell lines except HT1376 (Figure 2E–H). We also examined the expression of these two miRNAs in h-TERT, an immortalised normal urothelial cell line. Both miR-210 and miR-193b were upregulated by hypoxia in h-TERT cells, although the fold induction was lower than those observed in the bladder cancer cell lines (Supplementary Figure 2).
expression of miR-519a and miR-335 was unchanged by hypoxia (Supplementary Figure 3), whereas miR-518-3p was undetectable in these cells.

Using individual assays, the hypoxic induction of miR-518f, 200a, 15a, 99a, 107, 194 and 212, but not miR-150, was confirmed in T24 (Figure 4). In a second MI cell line, HT1376, miR-15a, 99a and 107 were unchanged by exposure to hypoxia, miR-194 and 212 were suppressed (Supplementary Figure 4) and miR-518f and miR-200a were undetectable.

Hypoxic upregulation of miR-145 requires HIF-1α but not p53. As mentioned previously, robust induction of miR-145 was observed in RT4 (Figures 1 and 5A ). MiR-145 was also induced upon treatment of cells with the hypoxia mimic DMOG in normoxia (Figure 5B). The upregulation of miR-145 in RT4 was of particular interest as miR-145 can, in part, be regulated by p53 (Sachdeva et al., 2009). As RT4 cells have wild-type p53, we investigated whether p53 was required for the hypoxic induction of miR-145. Knockdown of p53 did not reduce miR-145 expression in hypoxia (Figure 5C). However, knockdown of p53 attenuated the hypoxic induction of miR-210 (Figure 5D).

MiR-145 is a direct HIF-1α target gene. As the expression of miR-145 was induced by hypoxia and DMOG, we hypothesised that it was a direct HIF target gene in RT4 cells. Indeed, knockdown of HIF-1α but not HIF-2α attenuated the hypoxic induction of miR-145 (Figure 6A). A similar pattern of expression was observed for miR-210 (Figure 6B), a well-characterised HIF-1α target miRNA.

Using MatInspector (Cartharius et al., 2005), two putative HREs were identified in the promoter region of miR-145 (Figure 6C). To confirm that they were true HIF binding sites, ChIPs were performed with HIF-1α and RNA polymerase II antibodies. The HRE1, which is closer to the transcription start site (TSS) (Figure 6C), was enriched with both HIF-1α and RNA polymerase II antibodies (Figure 6D). The HRE2 that is 1.1 kb upstream of the TSS was only enriched with the HIF-1α antibody (Figure 6D). As a
positive control, the HRE of the robust HIF-1α target gene CA9 was enriched with both the HIF-1α and RNA polymerase II antibodies (Figure 6D) and the negative control UBC was not enriched in normoxia or hypoxia with either antibody (Figure 6D). Therefore, the hypoxic induction of miR-145 appears to be a direct effect of HIF-1α dependent transactivation.

MiR-145 regulates apoptosis under hypoxia in RT4 cells. As overexpression of miR-145 has been shown to affect cell viability in bladder cancer lines (Chiyomaru et al, 2010), we investigated whether miR-145 may play a role in cell viability in hypoxia in RT4 cells. Transfection of mimic-miR-145 in normoxia led to an increase in apoptotic annexin V+/PI+ cells and necrotic annexin V+/PI- cells compared with mimic-ctrl transfected cells and a concomitant decrease in viable annexin V+/PI- cells (Figure 7A and B and Table 1).

Exposure of anti-miR-ctrl transfected cells to hypoxia led to a decrease in cell viability as seen by an increase in necrotic annexin V+/PI+ cells and a decrease in viable annexin V+/PI- cells (Figure 7C and D and Table 1). Importantly, transfection of anti-miR-145 improved cell viability in hypoxia, with a decrease in annexin V+/PI+ cells and an increase in annexin V+/PI- cells (Figure 7E and Table 1). Thus, the hypoxic upregulation of miR-145 contributes to cell death under hypoxia in RT4 cells.

MiR-145 expression correlates with that of miR-210 and miR-193b in primary bladder cancer specimen. To determine the biological relevance of HRMs in vivo, we examined the correlation of miR-145, miR-210 and miR-193b expression in primary bladder cancer samples and in the normal bladder urothelium. The expression of miR-193b was strongly correlated to that of miR-145 (Figure 8A) and miR-210 (Figure 8B); the expression of miR-145 did not correlate to the expression of miR-210 in vivo (Figure 8C).

DISCUSSION

Variations in gene expression among different tumour types led us to hypothesise that exposure to hypoxia may lead to changes in miRNA expression that are unique to bladder cancer. Of the 25 miRNAs most highly induced in RT4 and T24, two cell lines that represent NMI and MI bladder cancer types respectively, the majority were exclusive to one or other. This is likely because of the differences between the molecular pathways involved in the two forms of bladder cancer that these two cell lines represent. Seven miRNAs were induced by hypoxia in both cell lines. They include the universal HRM miR-210 (Kulshreshtha et al, 2007; Camps et al, 2008) and miR-193b that is induced by hypoxia in the colon cancer line CaCo2 (Bruning et al, 2011). Thus, these may represent cell type-independent targets of HIF but further validation, particularly of miR-193b, is required. The fold induction of miR-210 in hypoxia was lower in the immortalised urothelium line h-TERT compared with the cancer-derived cell lines. This could reflect differences between cell lines or may suggest that the hypoxic response in cancer cells is augmented by additional pathways such as mTOR (Hudson et al, 2002).

Of note was the hypoxic induction of co-regulated miRNAs. Both miRNAs derived from pre-miR-125a, miR-125a-3p and miR-125a-5p (Jiang et al, 2010) were induced by hypoxia in RT4 and T24 cells. Furthermore, almost half of the 25 most hypoxia-induced miRNAs in RT4 cells, including miR-518a-3p, 515-5p and 525-3p, are from the primate-specific C19MC cluster on chromosome 19 (Bortolin-Cavaille et al, 2009). The induction of more than one mRNA from a precursor/cluster strengthens the notion that these are bona fide HRMs.

The hypoxic induction of miR-145, miR-125-3p, miR-708 and miR-517a was common to both NMI bladder cancer cell lines (RT4 and RT112). These four miRNAs, along with miR-210 and miR-193b, may form part of a HRM signature for NMI bladder cancer. Indeed, significant correlation was observed between miR-193b expression and that of miR-145 and miR-210 in NMI bladder cancer samples in vivo. In contrast, the majority of hypoxia-induced miRNAs in T24 cells could not be ratified in a second invasive bladder cancer line HT1376. Thus, MI bladder cancer-derived cell lines respond to hypoxia in a more varied manner, suggesting they are more divergent from each other.

Figure 7. Role of miR-145 in cell survival. RT4 cells were transfected with (A) mimic-ctrl and (B) mimic-145 and incubated in normoxia for 4 days. RT4 cells were transfected with (C and D) anti-miR-ctrl and (E) anti-miR-145 and incubated in (C) normoxia or (D and E) 0.1% O2 for 5 days. The x axis indicates PI staining and y axis indicates annexin V staining. (A–E) Data are representative of three independent experiments.
Members of the miR-200 family, associated with EMT, have been found to be downregulated in advanced bladder cancer (Wiklund et al., 2011).

In RT4 cells, the hypoxic induction of miR-145 was dependent on HIF-1α with two HREs identified in the promoter region. MIIR-145, thus, represents a new HIF-1α target gene in NMI bladder cancer lines and a novel HRM. The expression of miR-145 is known to be regulated by p53 (Sachdeva et al., 2009), thereby suppressing tumour growth and stem cell renewal. Conversely, overexpression of miR-145 suppresses growth and invasion in breast cancer cell lines (Kent et al., 2010; Sachdeva and Mo, 2010). Furthermore, miR-145 has been shown to lead to caspase-dependent and -independent cell death in bladder cancer cell lines (Chiyomaru et al., 2010; Ostenfeld et al., 2010; Noguchi et al., 2013). The miR-145 has also been shown to regulate PAI-1, an oncogene associated with a poor prognosis in bladder cancer, suggesting miR-145 may have a role as a prognostic indicator. In agreement with this, in this study, we have shown that increased levels of miR-145 in hypoxia contribute to cell death of RT4 cells. It is possible that one of the adaptive mechanisms in more malignant cancer types is the loss of hypoxic induction of miR-145, thus providing a survival advantage to cells.

In conclusion, we have shown that a relatively small-scale analysis can deliver novel insights into hypoxia biology. We have identified a number of miRNAs that could form part of a HRM signature, particularly in NMI bladder cancer. This strategy is capable of identifying direct HIF target genes, as demonstrated by miR-145. Finally, hypoxia-induced miRNAs are functionally relevant, as we have shown that miR-145 controls apoptosis in NMI bladder cancer cell lines. It may be worthwhile to validate the HRM signature identified herein in a larger cohort of NMI bladder cancer samples.

**ACKNOWLEDGEMENTS**

Work in this laboratory is supported by grants from Cancer research UK and the NHIR Biomedical Research Centre, Oxford. CB received grants from The Royal College of Surgeons of England, UCARE and The Urology Foundation. AR was funded by the Nuffield Dominon Trust.

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### Table 1. Percentage of cells in each quarter

|               | Mimic-ctrl N | Mimic-145 N |
|---------------|-------------|-------------|
| R3 (apoptotic)| 5.1         | 9.3         |
| R4 (necrotic) | 10.8        | 14.2        |
| R5 (viable)  | 74.5        | 67.5        |
| R6 (other)   | 9.5         | 9.0         |

|               | Anti-miR-ctrl N | Anti-miR-ctrl H | Anti-miR-145 H |
|---------------|----------------|----------------|---------------|
| R3 (apoptotic)| 3.6            | 2.4            | 3.5           |
| R4 (necrotic) | 15.2           | 35.1           | 23.4          |
| R5 (viable)  | 78.9           | 58.3           | 68.6          |
| R6 (other)   | 2.2            | 4.2            | 4.4           |

Abbreviations: ctrl – control; H – hypoxia; miR – microRNA; N – normoxia.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

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