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Hypoxia

miR-210 as a marker of chronic hypoxia, but not a therapeutic target in prostate cancer

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ABSTRACT

Introduction: Radiotherapy in combination with medical castration is the standard treatment for high-risk prostate cancer. Some relapses may be explained by the presence of radioresistant clones arising from hypoxic microenvironment. Since microRNAs (miR) are increased upon hypoxia, the aim of this study was to see whether miR-210 is a potential marker for hypoxia and/or a therapeutic target in prostate cancer.

Methods: Human LNCaP, DU145 or PC3 prostate cancer cells were exposed to normoxia or hypoxia for several hours. Gene expression of miR-210, miR-373 and several hypoxia markers were analyzed by Taqman and SYBR green qRT-PCR, respectively. Clonogenic survival after LNA miR-210 inhibitor (78 nM) and concomitant irradiation were evaluated.

Results: During anoxia, CAIX and VEGF expressions were dramatically increased. miR-210 expression increased during anoxia exposure, while basal miR-373 expression was low and remained stable upon anoxia. LNA miR-210 inhibitor decreased anoxic miR-210 expression by 90% and clonogenic survival under anoxia ($p = 0.01$). However, no enhanced effect was observed when miR-210 inhibitor was combined with irradiation.

Conclusion: miR-210 could be an interesting marker of chronic hypoxia irrespective of the androgen dependency and should, therefore, be tested as a prognostic marker in high risk prostate cancer patients.

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In Europe, prostate cancer is the leading cause of cancer incidence and mortality in men with nearly 346,000 and 87,400 cases, respectively, in 2006 [1]. Radiotherapy with or without medical castration using LHRH (luteinizing hormone releasing hormone) agonists is one of the standard treatments of localized prostate cancer. Unfortunately, as much as 21% of patients may relapse clinically after treatment [2]. Treatment failure depends on several parameters such as number of clonogenic cells, the tumor growth rate, the cell repair capacity, the intrinsic cellular radiosensitivity and the tumor microenvironment. Tumor hypoxia or hypoxia response could also be an explanation for treatment failure after radiotherapy. Indeed, hypoxia is associated with poor disease-free survival after radiotherapy in many cancers, such as head and neck [3] and cervical cancers [4]. In localized prostate cancer, tumor hypoxia is associated with poor prognosis, since low pO2 levels, measured by Eppendorf microelectrode in prostate, was associated with higher biochemical failure assessed by serum PSA (prostate specific antigen) concentrations [5]. Furthermore, a significant correlation was found between pre-operative prostate hypoxia level measured by eppendorf oxygen microelectrodes and percentage of cells staining positive for VEGF [6]. In an ancillary study from the randomized trial GETUG-06 (French Genital and Urinary Tumors Study Group), high expression of hypoxia markers HIF-2α and CAIX, has been associated with poor biochemical control after radiotherapy [7].

Two types of tumor hypoxia are recognized. Chronic hypoxia results from the limited diffusion distance of oxygen in tissue, whereas acute or transient hypoxia is caused by local fluctuations in tumor blood perfusion [8]. Acute/cycling hypoxia has been described as a major source of therapy resistance [9–11].

MicroRNA (miR) are small non-coding RNA, 20–22 nucleotides in length, that regulate gene expression at post-transcriptional levels. They act by blocking translation or inducing degradation of the target mRNA after hybridization to its 3′-untranslated region (UTR) [12]. Actually, >1000 miR have been identified in humans and they are involved in the regulation of at least one third of all translated genes. MicroRNA are implied in the regulation of many cell processes such as metabolism, differentiation, proliferation [13] and death [14]. miR-210 is the microRNA most frequently identified in response to hypoxia and has been identified in a microarray analysis as the predominant miR induced by hypoxia in pancreatic cancer cell lines as its induction was dependent on HIF-1α [15].

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This induction of miR-210 under hypoxia was confirmed in other cell lines, such as breast, head and neck, lung, colon, and renal cancer cell lines [16]. Recently, miR-210 have been associated with poor prognosis in early breast cancer in which miR-210 levels above the median associated with lower survival at 10 years [17]. Additionally, microarray analysis revealed that hypoxia induced not only miR-210, but also miR-373 in Hela cells (8.4 and 5.6-fold increase, respectively) [18].

In this study, we hypothesized that miR-210 or miR-373 expression in human prostate cancer cell lines would correlate with hypoxia and radiosensitivity. To validate miR-210 or miR-373 as a hypoxic marker, we have examined and correlated their expression with other well-known tumor hypoxia markers. Furthermore, to investigate their therapeutic properties, we have examined cell survival after irradiation in combination with miR inhibition upon hypoxia.

Materials and methods

Cell culture and hypoxia treatment

The human cancer prostate cell lines LNCaP, DU145, and PC3 were purchased from American Type Culture Collection (ATCC; Manassas, VA). Cells were grown as monolayer, in RPMI-1640 (LNCaP, PC3) and McCoy-5A medium with glutamine, supplemented with 10% fetal calf serum. Cells were plated at a density of 5×10^5 per 6 cm dish a day before the start of the experiment and transferred to a hypoxic culture chamber (MACS VA500 microaerophilic workstation, Don Whitley Scientific, Shipley, UK). The atmosphere in the chamber consisted of 0% O_2, 5% CO_2, 5% H_2 and residual N_2. Normoxic dishes were incubated in parallel in air with 5% CO_2.

MicroRNA qRT-PCR

Total RNA from cultured cells was extracted with TRizol reagent (Invitrogen, Breda, The Netherlands) as previously described [19]. Total RNA (10 ng) was transcribed using TaqMan microRNA reverse transcription kit (Applied Biosystems) according to manufacturer's instructions. Products were amplified by PCR using TaqMan Universal PCR Master Mix kit (Applied Biosystems). Primers were purchased from Applied Biosystems: hsa-miR-210 (CUGUGCGUG UGACAGCGGCUGA), hsa-miR-373 (GAAGUGCUUCGAUUUUGGG-GUGU). Values for each miR were normalized to expression levels of microRNA RNU6B (CGCAAGGAUGACACGCAAAUUCGUGAAGC-GUUCCAUAUUUUU). Real-time PCR was performed in ABI 7500 (Applied Biosystems).

mRNA qRT-PCR

Total RNA (1 μg) was transcribed using iScript cDNA synthesis kit (BioRad Laboratories). cDNA was then amplified using SYBR Green Master Mix (Applied Biosystems). Values for each gene were normalized to expression levels of GAPDH RNA. Following primers,

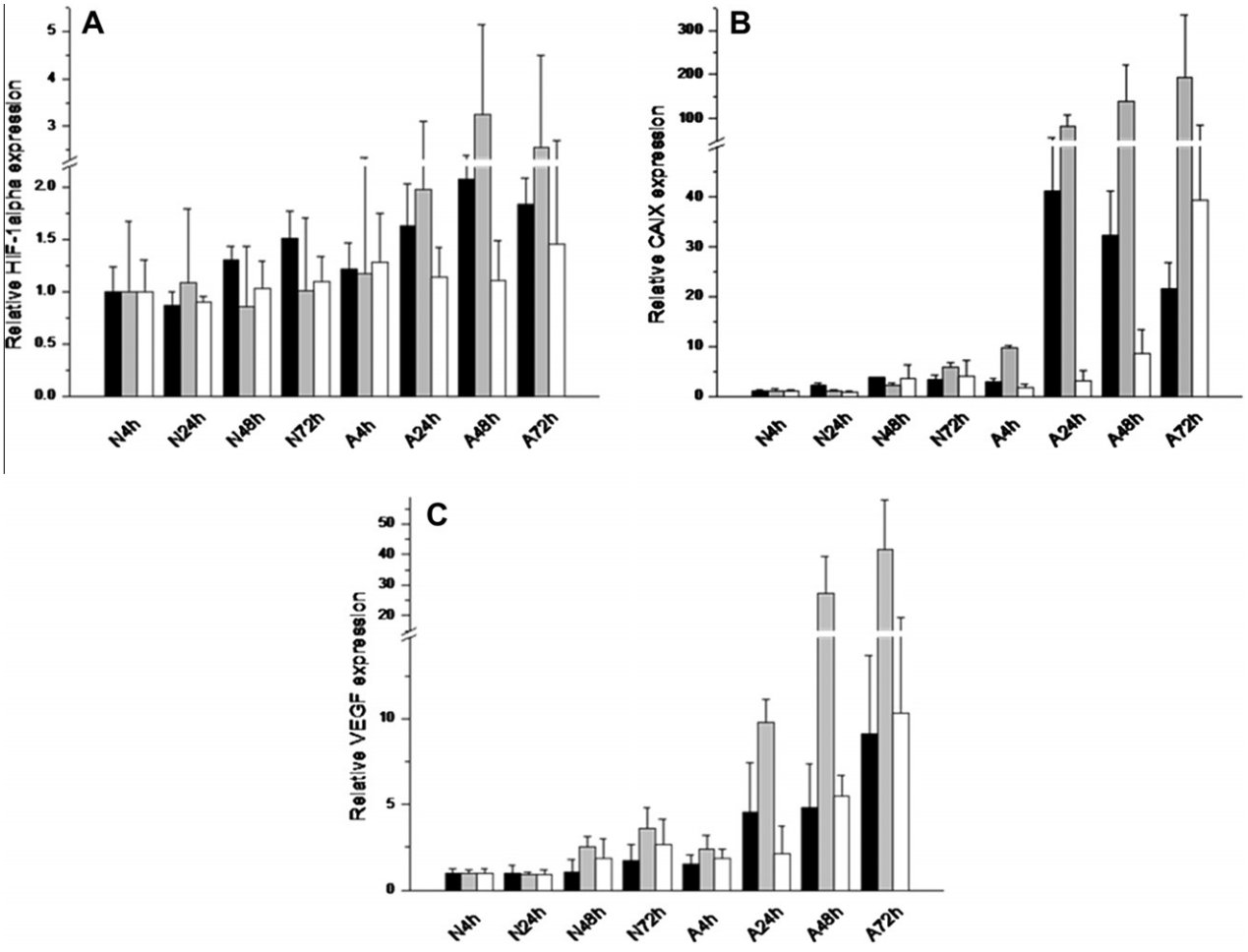

Fig. 1. Quantitative real-time PCR of HIF-1α (A), CA IX (B) and VEGF (C) mRNA in PC3 (black), LNCaP (gray) and DU145 (white) human prostate cancer cell lines in response to normoxia or anoxia for the indicated time points. All results are normalized to GAPDH mRNA expression. Data are represented as the mean ± SD of three independent experiments.

specific for the hypoxia response genes HIF-1α, CAIX and VEGF were used: HIF-1α (F-ATCGCGGGGACCGATT and R-CGACGTTCA-GAACTTATCTTTTTCTT), CAIX (F-ATCCTAGCCCTGGTTTTTGG and R-GCTCACACCCCCTTTGGTT), VEGF (F-GACTCCGGCGGAAGCAT and R-TCCGGGCTCGGTGATTTA), GAPDH (F-GCACCACCAACTGCTTAGCA and R-TGGCAGTGATGGCATGGA) and 18S rRNA (F-AGTCCCTGC CCTTTGTACACA and R-GATCCGAGGGCCTCACTAAAC).

Transfection with miR-210 inhibitor

miR-210 inhibitor was purchased from Exiqon company (Vedbæk, Denmark) and was based on miRCURY LNA™ microRNA Knockdown technology. Cells were transfected 10 min before hypoxia treatment with miR-210 inhibitor (final concentration of 78 nM) using lipofectamine 2000 (Invitrogen, Breda, The Netherlands) according to the manufacturer's recommendations. Control consisted of scramble miR inhibitor 3′-fluorescein labeled (Exiqon, Vedbæk, Denmark). The sequences of the miR-210 inhibitor and the scramble miR were 5′-TCAGCCGCTGTCACACGCACAG-3′ and 5′-GTGTAACACGTCTATACGCCCA-3′, respectively. Transfection efficiency was evaluated by fluorescence microscopy (Leica Microsystems).

Cell irradiation

Irradiation was performed using a MCN 225 Philips 225 kV X-ray irradiator (Philips, Eindhoven, Netherlands), at a dose rate of 0.7 Gy/min. During irradiation, cells were placed on ice into air-tight sealed chambers to avoid modification of oxygen concentration.

Clonogenic survival assay

Cells were exposed for 10 min to miR210 inhibitor or scramble miR before exposure to anoxia or normoxia for 24 h. After irradiation, cells were trypsinized and plated in triplicate for clonogenic survival. Cells were allowed to form colonies during 14 days before fixation and staining with 0.2% methylene blue in 70% ethanol. Colonies containing more than 50 cells were scored. Surviving fraction was calculated as the ratio between the number of counted colonies and the number of plated cells, corrected for plating efficiency (PE).

Statistics

All statistical analyses were performed with SPSS 12.0.1 for Windows (SPSS Inc., 2003, Chicago, USA). A $p < 0.05$ was considered significant.

Results

First we examined the hypoxia responsiveness of the three different prostate carcinoma cell lines by assessing the HIF-1α, CA IX and VEGF gene expression upon anoxic exposure relative to the normoxic control conditions. Anoxia resulted in an increased

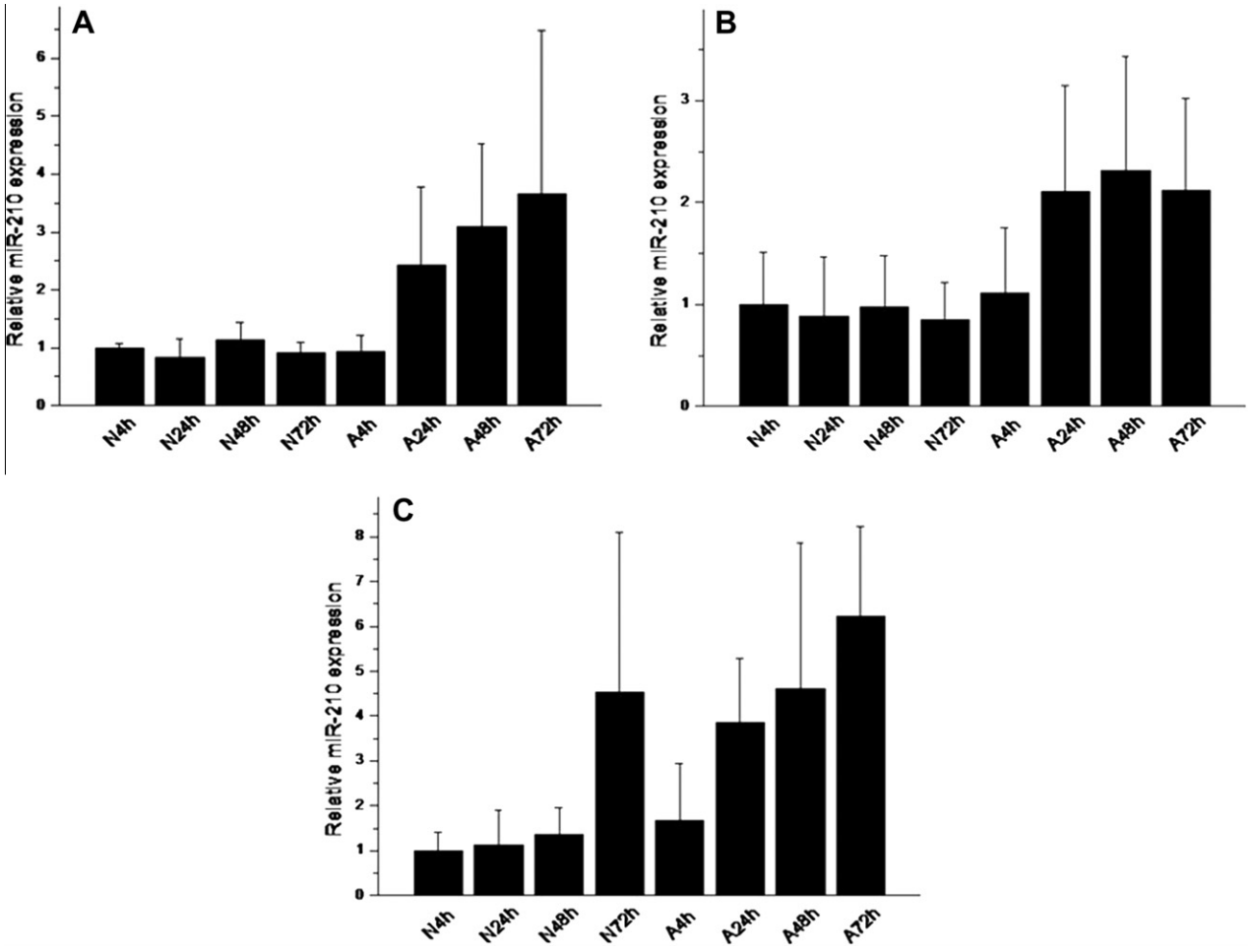

Fig. 2. Quantitative real-time PCR of miR-210 expression in PC3 (A), LNCaP (B) and DU145 (C) human prostate cancer cell lines in response to normoxia or anoxia for the indicated time points. All results are normalized to microRNA RNU-6B expression. Data are represented as the mean ± SD of two independent experiments.

HIF-1α expression reaching a plateau at 48 h exposure for the PC3 and LNCaP cell lines, while no increase was observed for the DU145 (Fig. 1A). In general, the increased HIF-1α expression was higher in the androgen dependent LNCaP prostate cell line. Corresponding with the increased HIF-1α expression, the expression of the HIF-responsive downstream targets CAIX and VEGF was also increased upon anoxic exposure, again with higher expression levels in the androgen dependent cell line (Fig. 1B and C). Surprisingly, although no increased HIF-1α expression was observed in the DU145 cells, CAIX and VEGF expression levels were increased. GAPDH expression was not significantly altered upon anoxia, in all three prostate cancer cell lines, in comparison with the house keeping gene 18S rRNA expression (data not shown).

Next, we investigated how anoxia influences miR-210 expression. Growth under normoxic conditions did not seem to influence miR-210 expression levels (Fig. 2), even not at higher cell densities (72 h time point), except for the DU145 cells. However, this 72 h data point should be taken with caution (very large standard deviation). Anoxia exposure resulted in an increased miR-210 expression for all investigated cell lines, with a preference for the androgen-independent cell lines DU145 and PC3 (Fig. 2), indicating that miR-210 is a more stable surrogate chronic hypoxia marker in prostate cell lines.

miR-210 expression was significantly correlated with CA IX and VEGF mRNA expressions, in androgen-independent cell lines, with respectively: $p = 0.03$ and 0.002 (DU145) and $p = 0.001$ and 0.02 (PC3). Only miR-210 and VEGF mRNA expression was significantly correlated in the androgen-dependant cell line LNCaP ($p = 0.002$).

Additionally, we also investigated miR-373 levels under anoxia. Anoxia exposure did not seem to influence miR-373 expression in all cell lines tested (Fig. 3). miR-373 expression levels were extremely low both in normoxic and anoxic conditions (Ct-values range between 34 and 40).

To examine miR-210 as a potential therapeutic target in prostate cancer cells, we exposed PC3 cells to 78 nM LNA miR-210 inhibitor during 24 h under anoxia. The increased miR-210 expression of a scrambled inhibitor upon anoxic exposure could be rescued with a specific miR-210 inhibitor (Fig. 4A). The difference in miR-210 expression after miR-210 specific inhibitor treatment was statistically significant compared with the scramble control inhibitor ($p = 0.03$). We observed a dose–response relationship between miR-210 inhibitor concentration during anoxia and inhibition of miR-210 expression in PC3 cell line. Furthermore, miR-210 inhibition decreased the anoxia tolerance to approximately 72% (Fig. 4B), indicating an unexpected statistically significant mortality upon miR-210 inhibition ($p = 0.01$).

Based on these results, we investigated if this decreased anoxia tolerance could lead to an increased sensitization of prostate cells to irradiation. PC3 cells were exposed to miR-210 inhibition during 24 h under anoxia and cells were afterward exposed to a range of irradiation doses. Cells incubated with a scrambled control inhibitor showed a significant resistance phenotype when exposed to anoxia, the oxygen enhancement ratio (OER) was calculated to be 2.0 ± 0.3 (mean ± SD). Incubation of cells to a specific miR-210 inhibitor under normoxic conditions had no influence on radiosensitivity (Fig. 5). Similar data were found upon incubation under

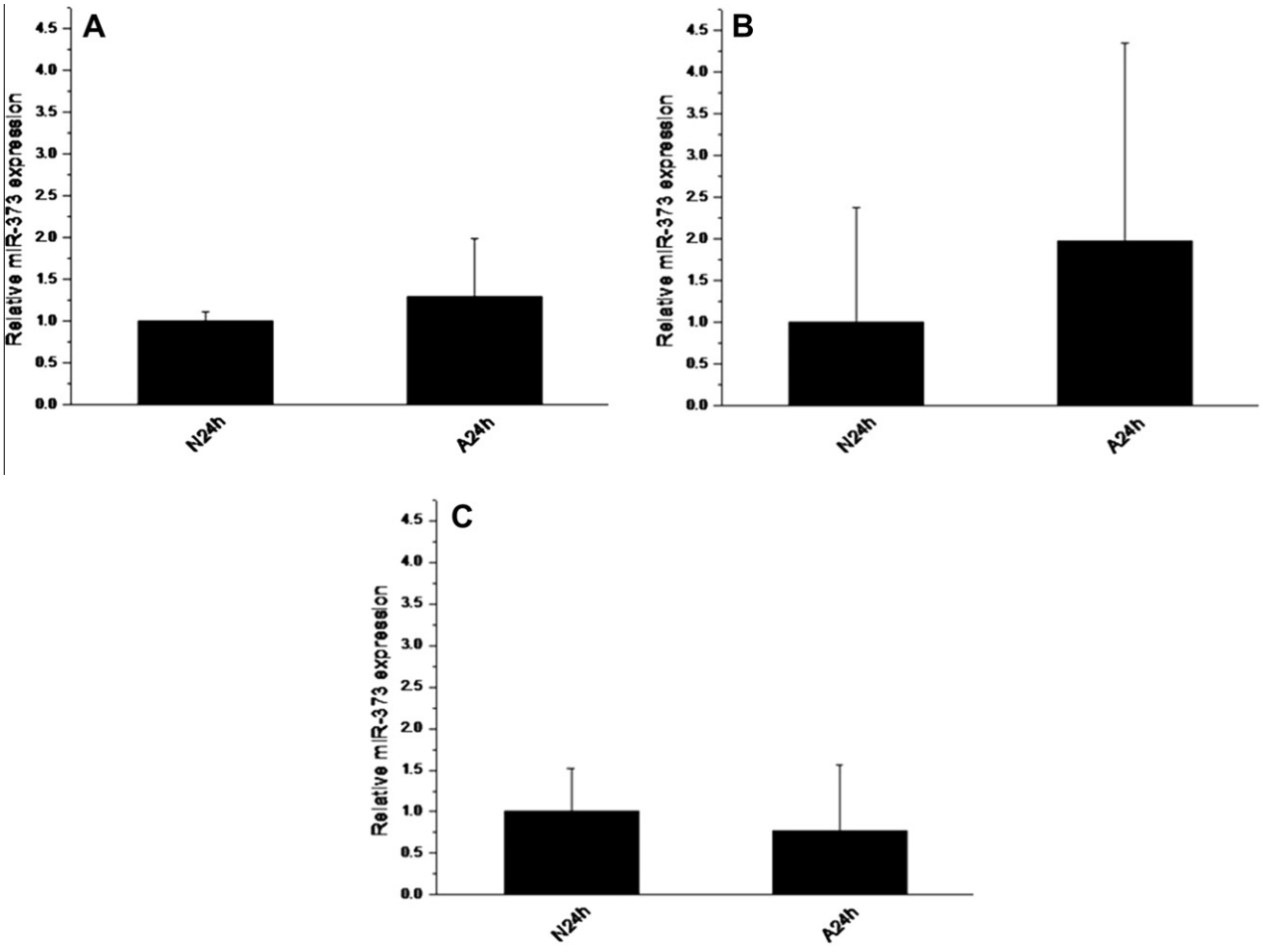

Fig. 3. Quantitative real-time PCR of miR-373 expression in PC3 (A), LNCaP (B) and DU145 (C) human prostate cancer cell lines in response to normoxia or anoxia for the indicated time points. All results are normalized to microRNA RNU-6B expression. Data are represented as the mean ± SD of two independent experiments.

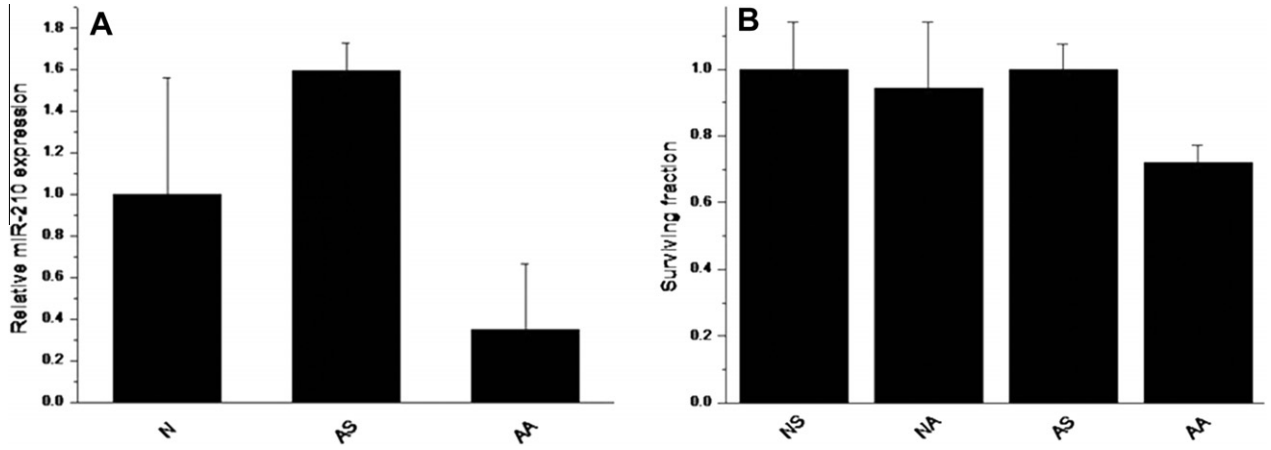

Fig. 4. (A) Quantitative real-time PCR of miR-210 expression in PC3 cells after addition of 78 nM specific miR-210 (A) or scrambled (S) inhibitor during 24 h normoxia (N) or anoxia (A). Data are normalized to microRNA RNU-6B expression. (B) Clonogenic survival of PC3 cells exposed to 78 nM specific miR-210 (AA) or scrambled (AS) inhibitor during 24 h anoxia. Data are represented as the mean ± SD of three independent experiments.

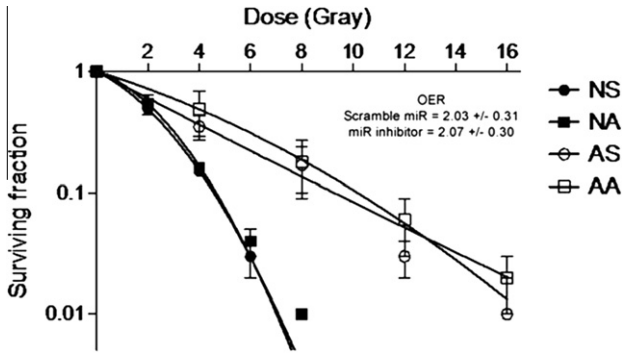

Fig. 5. Clonogenic survival of PC3 cells after irradiation under normoxic (black) and anoxic (white) conditions. Cells were pre-incubated under the respective conditions with 78 nM specific miR-210 (squares) or scrambled (circles) inhibitor. Data are represented as the mean ± SD of three independent experiments. Oxygen enhancement ratio (OER) values for scramble miR and miR-210 inhibitor.

anoxic conditions, indicating that miR-210 inhibition could not result in a radiosensitization upon anoxia.

Discussion

Hypoxic tumors are associated with poor prognosis and resistance to radiotherapy [3–5,20–22]. For prostate cancer, tumor hypoxia is associated with tumor aggressiveness, high histological grade and advanced clinical stage [6,23,24]. Increased hypoxia marker expression is associated with poor disease control after radiotherapy. Elevated HIF-1α and VEGF expressions on tumor biopsies from patients treated for prostate cancer by radiotherapy have been associated with poor biochemical control of the disease [25]. In our study, all three investigated cell lines proved to be hypoxia-responsive as evidenced by increased HIF-1α, CAIX and VEGF mRNA expression. Although we did not observed any HIF-1α mRNA induction in DU145 after anoxia, CAIX and VEGF expression levels were increased which could be explained by post-translational regulation of HIF-1α [26].

miR-210 is the microRNA most frequently associated with tumor hypoxia [16,17,27] and increased miR-210 expression was observed in vitro after 24 h exposure to hypoxia in pancreatic, breast, head and neck, lung, colon, and renal cancer cell lines [15]. So far, it was not known if miR-210 follows the same pattern in prostate cancer cell lines exposed to low oxygen concentrations. In our study, we observed miR-210 expression consistently increased upon anoxia exposure in prostate cancer cell lines irrespective of the androgen dependency. Furthermore, miR-210 upregulation was more consistently associated with chronic hypoxia across prostate cancer cell lines compared to CA IX, VEGF and HIF-1α. This was somewhat anticipated since several studies have shown that miR-210 was a target of HIF-1α [16,17,27]. A functional hypoxia-responsive element (HRE) on the genomic DNA, upstream of the miR-210 coding gene, confirming miR-210 was regulated by HIF-1α [15]. Our results are furthermore in accordance with the observed association between HIF-1α, GLUT-1 expression and miR-210 expression in primary tumor samples from patients with head and neck cancer [15]. These data suggest that miR-210 expression might be a valuable surrogate marker for chronic hypoxia, a type of hypoxia potentially easier to tackle with radiation in contrast with acute hypoxia. Additionally, recent studies concluded that analysis of circulating miR-210 could be used as a cancer biomarker in large B-cell non Hodgkin lymphoma [28] and pancreatic cancer [29]. Circulating miR210 analysis is a promising technique because it is a non-invasive method permitting repeated measurements over time.

Although it has been demonstrated that miR-210 and miR-373 was up-regulated upon hypoxia exposure in cervical and breast cancer cell lines [18], we only observed an increased miR-210 expression upon anoxia, while no significant miR-373 expression in the prostate cancer cell lines. miR-373 expression levels were generally low and Ct values ranged from 34 to 40. In the previous report, we could not find any information about Ct values, but our results seem to be in accordance with Schaefer et al., who could not detect any miR-373 expression in RT-qPCR in prostate cancer tissues (Ct values >35) [30]. These findings indicate that only miR-210 and not miR-373 could be used a marker for chronic hypoxia.

Several miR-210 targets have been identified such as HOXA1 (effect on cell proliferation and cell oncogenic transformation), FGFRL1 (effect on cell proliferation), HOXA9 (effect on cell proliferation and apoptotic cell death) [15] and RAD52 (effect on DNA repair through homologous recombination) [18]. Based on the functions of these targets, we hypothesized that miR-210 might be a potential therapeutic target. miR-210 inhibition was effective under anoxia and resulted in a decreased clonogenic survival relative to a control inhibitor. These results prompted us to investigate whether miR-210 inhibition would enhance the effect of radiotherapy. As expected, a radioresistant phenotype was observed in our prostate cancer cell line model after anoxia exposure relative to normoxia.

Oxygen enhancement ratio (OER) was lower than expected: 2 for PC3 cell line in this study vs 3 for most cells. Reduced OER under chronic hypoxia (>24 h) was also observed in PC3 by Stewart et al. [31] and was most common in p53 mutant cell lines such as PC3 cell line [32,33].

However, pretreatment with miR-210 inhibitors did not sensitize cells to irradiation, neither under normoxia nor upon anoxia, indicating that miR-210 is not a therapeutic target. Possibly, downstream miR-210 targets such as RAD52 are more suited to inhibit, which is involved in homologous recombination and has a direct effect on radiation efficacy.

Conclusion

miR-210 might be an interesting marker of chronic hypoxia irrespective of the androgen dependency and should, therefore, be tested as a prognostic marker in high risk prostate cancer patients. Our results need to be confirmed in vivo on primary tumor sections with comparison to HIF-1α, CA IX and VEGF expressions. Analysis of circulating miR-210 could be an alternative to primary tumor staining and could be tested in patients with prostate cancer as a biomarker. Although inhibition of miR-210 on its own results in a higher cell death upon anoxia, no extra-therapeutic gain was obtained for concomitant association with radiotherapy.

Conflict of interest statement

The authors declare that they have no conflict of interest related to the contents of this manuscript.

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