RESEARCH COMMUNICATION

MicroRNA-100 Acts as a Tumor Suppressor in Human Bladder Carcinoma 5637 Cells

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Abstract

Bladder carcinoma is one of the most common tumors in the world and, despite the therapy currently available, most of the patients relapse. Better understanding of the factors involved in disease pathogenesis would provide insights for the development of more effective strategies in treatment. Recently, differential miRNA expression profiles in bladder urothelial carcinomas identified miR-100 down-regulation and miR-708 up-regulation among the most common alterations, although the possible influence of these miRNAs in the control of basic mechanisms in bladder tumors has not been addressed. In this context, the present study aimed to evaluate the in vitro effects of miR-100 forced expression and miR-708 inhibition in the bladder carcinoma cell line 5637. Our results showed that overexpression of miR-100 significantly inhibited growth when compared to controls at both times tested (72 and 96 hours, $p<0.01$) with a maximum effect at 72 hours reducing proliferation in 29.6 %. Conversely, no effects on cell growth were observed after inhibition of miR-708. MiR-100 also reduced colony formation capacity of 5637 cells by 24.4%. No alterations in cell cycle progression or apoptosis induction were observed. The effects of miR-100 on growth and clonogenicity capacity in 5637 cells evince a possible role of this miRNA in bladder carcinoma pathogenesis. Further studies are necessary to corroborate our findings and examine the potential use of this microRNA in future therapeutic interventions.

Keywords: MicroRNA-100 - tumor suppressor - bladder carcinoma

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Introduction

Bladder carcinoma is one of the most common tumors in the world and, despite the therapy currently available, 50-70% of the patients with advanced forms relapses in less than 5 years and succumb to the disease (Jacobs et al., 2010). The mechanisms of bladder tumorigenesis are not totally recognized, therefore, better understanding of the factors involved in tumor progression is important for the development of more effective treatment strategies.

In this context, the importance of studying microRNAs arises. These are small (17-25 nucleotides) single-stranded RNAs that function predominantly as sequence-targeted modifiers of gene expression through translational repression (Filipowicz et al., 2008; Almeida et al., 2011). More than 1,000 miRNAs have been described so far for the human genome with thousands of predicted target mRNAs that participate in a wide variety of physiological processes such as cell cycle progression, apoptosis and differentiation, though many of them still do not have well established functions (Lu et al., 2005; Nana-Sinkam, Croce, 2011).

A recent article published in the Asian Pacific Journal of Cancer Prevention on differential miRNA expression profiles in bladder urothelial carcinomas, identified miR-100 down-regulation and miR-708 up-regulation among the most common alterations found in these tumors compared to the adjacent normal counterparts (Song et al., 2010). Though, the possible influence of these miRNAs in the control of basic mechanisms in bladder tumors has not been addressed. In this context, the present study aimed to evaluate *in vitro* effects of miR-100 forced expression as well as miR-708 inhibition in 5637 bladder tumor cell line.

Materials and Methods

Cell Culture

The established bladder carcinoma cell line 5637 (moderately differentiated tumour) was obtained from the Cell Bank of the Federal University of Rio de Janeiro, Brazil. Cells were cultured in RPMI 1640 (GibcoBRL, Life Technologies®, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum, penicillin (100U/mL) and streptomycin (100ug/mL) at 37°C in a humidified 5% CO2 incubator.

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Transfection of microRNAs

Pre-microRNA miR-100 and negative control (Ambion Pre-miR miRNA Precursors®, AB) and anti-microRNA miR-708 and negative control (Ambion Anti-miR miRNA Inhibitors®, AB) were reverse-transfected into bladder cell lines using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at a final concentration of 100 nM. The microRNAs transfection efficiency was monitored by qRT-PCR at 24, 48 and 72hr.

RNA extraction, cDNA synthesis and quantitative real-time PCR (RQ-PCR)

Total cellular RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The RNA was stored in DEPC-treated water at -80ºC and, before use; the quantity and quality of samples were evaluated by ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies). 100 ng of total RNA was retro-transcribed with microRNA-specific primers using a TaqMan microRNA reverse transcription kit (Applied Biosystems, Foster City, CA), and qRT-PCR was then performed using Taqman® microRNA assays according to the manufacturer's protocol.

The microRNA levels were measured using the ABI 7500 Real Time PCR System (PE Applied Biosystems). The relative expression was calculated using the 2-ΔΔCT method (Livak, Schmittgen, 2001) with two internal controls, small nuclear RNU6B and RNU48. The expression levels in negative controls were used as calibrator.

Real time PCR was performed in duplicate and a standard deviation (SD) of <0.5 between duplicates was accepted. A blank control was run in parallel to determine the absence of contamination within each experiment.

Measurement of cell growth

Cell survival was assessed using the XTT assay (XTT II; Roche Molecular Biochemicals, Indianapolis, IN). Briefly, 24hr after transfection, the cells were seeded in 96-well flat-bottom plates (2500 cells/well) and were then incubated for 72h and 96h after transfection. After those periods the culture medium was removed and replaced with medium containing 10 μL of XTT dye (3 mg/mL) in each well. The plates were incubated for 2 hours at 37°C and the formazan product was measured at 455 and 650 nm by using an iMark microplate reader (Bio-Rad Laboratories®). Each experiment was performed in triplicate wells and repeated in three sets of tests.

Colony Formation Assay

Clonogenic assays were performed according to Franken et al., 2006. After tripsinization, single cell suspensions of 200 cells were seeded in 6-well plates after 24 hr of transfection. The cell cultures were incubated for 10 days. Colonies were then rinsed with PBS, fixed with methanol and stained with Giemsa. The colonies with > 50 cells were counted. Assays were performed in triplicate.

Detection of apoptotic cells

For apoptosis 3x104 cells were seeded on 6-well plates containing 3 mL of culture medium after 24 hr after treatment. After 96hr, Caspase activity was measured through the NucView™ 488 Caspase-3 Detection in Living Cells kit (Biotium Inc. Hayward, CA, USA) according to the manufacturer's instructions. Concisely, transfected cells were trypsinized and incubated for 40 minutes at room temperature with the caspase-3 substrate. Then cells were fixed in formaldehyde, counterstained with 4',6-diamidino-2-phenylindole (DAPI), mounted, coverslipped and analyzed by fluorescence microscopy with a triple filter. Five hundred nuclei were analyzed per treatment and cells were scored and categorized according to differential staining.

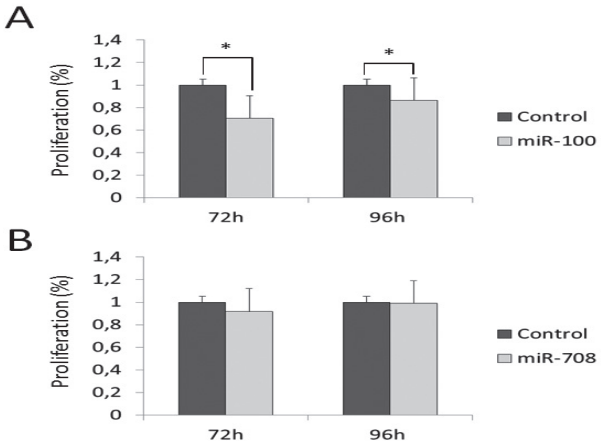

Figure 1. Proliferation Data. A) Decreased Proliferation Rate in 5637 Cell Lines Treated with Pre-microRNA miR-100 Analyzed After 72h and 96h; B) Apparently No Difference in Proliferation Rate in 5637 Cell Lines Treated with Anti-microRNA miR-708 Analyzed After 72h and 96h

Cell cycle analysis

After 72, 96 and 120 hr of transfection, cells were fixed in 70% ethanol, stained with propidium iodide, and analyzed on a Guava Personal Cell Analysis system (Guava Technologies, Hayward, CA, USA) according to the standard protocol provided by the manufacturer. Percentages of cells in G0/G1, S, or G2/M phase were collected and processed using the GUAVA Cytosoft 4.2.1 version Software.

Statistical analysis

Data was statistically analyzed by Student's two-tailed t-test using the Statistical Package for the Social Sciences (SPSS) software for Windows, version 15.0 (SPSS Inc., Chicago, IL, USA). All tests were carried out for $\alpha = 0.05$.

Results

MiR-100 inhibits cell proliferation in vitro

MiR-100 significantly inhibited growth of 5637 cells when compared to control at both times tested (72 and 96 hours, $p<0.01$) with a maximum effect at 72 hours reducing proliferation in 29.57 % (Figure 1A). However, this effect was not observed after inhibition of miR-708 (72 hours, $p=0.14$; 96 hours, $p=0.76$) (Figure 1B).

MiR-100 reduces the clonogenic capacity of 5637 cells

MiR-100 significantly reduced the colony formation

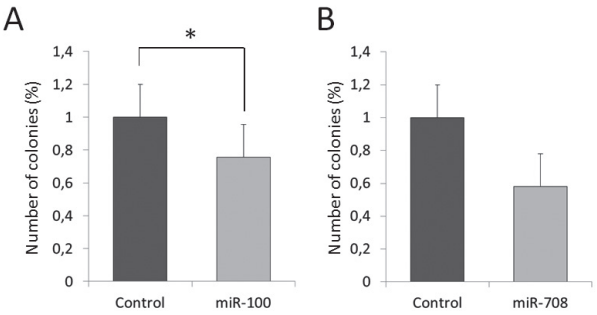

Figure 2. Clonogenic Capacity. A) Decreaseof 5637 Cell Lines After Treatment with Pre-microRNA miR-100; B) Although Lower, the Clonogenic Capacity of 5637 Cell Line Was Not Significantly Altered After Treatment with Anti-microRNA miR-708.

Table 1. Cell Cycle Analysis of 5637 Cell Line Treated with Pre-microRNA miR-100 and anti-microRNA miR-708

	96h	120h
SUB G1 (%)		
Pre-miR negative control	13.3 ± 4.34	14.2 ± 7.87
Pre-microRNA miR-100	8.69 ± 4.20	7.05 ± 4.46
Anti-miR negative control	5.85 ± 2.44	5.22 ± 3.45
Anti-microRNA miR-708	5.85 ± 1.88	6.22 ± 5.54
G1 (%)		
Pre-miR negative control	47.2 ± 5.25	48.1 ± 9.18
Pre-microRNA miR-100	47.7 ± 7.63	51.1 ± 4.23
Anti-miR negative control	45.5 ± 5.09	56.1 ± 2.19
Anti-microRNA miR-708	46.7 ± 2.26	55.7 ± 2.77
S (%)		
Pre-miR negative control	16.8 ± 4.71	13.9 ± 2.65
Pre-microRNA miR-100	14.7 ± 2.70	17.0 ± 1.74
Anti-miR Negative control	13.4 ± 2.11	15.5 ± 0.95
Anti-microRNA miR-708	14.2 ± 2.23	13.9 ± 2.32
G2 (%)		
Pre-miR negative control	24.4 ± 6.50	24.9 ± 3.51
Pre-microRNA miR-100	29.4 ± 9.31	25.8 ± 2.94
Anti-miR negative control	36.4 ± 3.66	24.3 ± 5.99
Anti-microRNA miR-708	33.4 ± 0.99	25.3 ± 5.03

Percentages of Cells in G1, S, and G2/M phases are expressed as mean ± standard deviation

capacity of 5637 cells when compared to control (p=0.03) (Figure 2A), being 24.4 % lower after treatment. In the case of miR-708 the clonogenic capacity was also reduced although in this case no statistical differences were observed (p=0.13; Figure 2B).

MiR-100 and miR-708 do not induce apoptosis

To determine the induction of apoptosis in 5637 cells treated with miR-100 or miR-708, caspase-3 activity was measured. Compared to controls, neither of the microRNAs studied was found to induce apoptosis in the treated 5637 cells (p=0.37 and p=0.85, respectively; data not shown).

Transfection with MiR-100 and miR-708 do not alter cell cycle progression in 5637 cell lines

When compared to control, the distributions of cells along cell cycle phases were not altered after transfection (Table 1).

Discussion

Low miR-100 expression has been described as an important miRNA alteration in bladder tumors (Ichimi et al., 2009, Song et al., 2010), but its possible influence in tumor pathogenesis has not been evaluated yet. In the present study, forced expression miR-100 decreased cell proliferation and colony formation capacity of human bladder cancer 5637 cells. These results are in accordance with previous reports of significantly decreased proliferation rates in oral squamous carcinoma (OSCC) and cervical cancer cell lines after transfection with this microRNA (Henson et al., 2009; Li et al., 2011), pointing to the possibility of miR-100 hypoexpression having important roles in maintenance of high growth rates in cancer.

This influence of miR-100 in proliferation control is also reinforced by the identification of protein targets that are known to play important roles in the regulation of cellular growth, proliferation and survival, for example such as PLK1 and FRAP1/mTOR (Nagaraja et al., 2010; Shi et al., 2010; Tovar et al., 2010). Hyperexpression of PLK1 has been demonstrated in bladder tumors (Nogawa et al., 2005; Yamamoto et al., 2006) and this could be an important factor associated with tumor progression and prognosis. Additionally, a substantial number of articles have provided evidence of high activation of mTOR pathways associated with decreased proliferation rates after treatment with different inhibitors (Garcia et al., 2008; Park et al., 2011; Schedel et al., 2011). Moreover, the FGFR3 protein, a growth factor frequently mutated in low-grade urothelial cell carcinoma, has been experimentally validated as target of miR-100 in bladder cells (Catto et al., 2009).

Considering miR-708 inhibition, the present study was unable to confirm any possible effects of this miRNA in proliferation, apoptosis nor cell cycle regulation.

In the literature, there is a single evidence of miR-708 influencing biological parameters. This microRNA is reported to be hypoexpressed in renal cell carcinoma, and its forced expression results in decreased cell growth, clonogenicity and a dramatic increase of apoptosis (Saini et al., 2011).

Opposite differential expression patterns for miR-708 have been demonstrated in other tumors, being hyperexpressed in bladder (Song et al., 2010) and colon tumors as well as B-cell acute lymphoblastic leukemias (Necela et al., 2011; Shotte et al., 2009), although, its expression has been indicated as a good prognosis marker in lung tumor (Patnaik et al., 2010, Xing et al., 2010). Yet, the role of miR-708 expression in bladder carcinoma, remains obscure and further studies are necessary in the field.

In conclusion, the present study demonstrated effects of miR-100 in growth cell rates and clonogenicity capacity in 5637 cell line, emphasizing a possible effect of this miRNA in bladder carcinoma pathogenesis. Further studies are necessary to corroborate our findings and examine the potential use of this microRNA in future therapeutics interventions.

References

Almeida MI, Reis RM, Calin GA (2011). MicroRNA history: Discovery, recent applications, and next frontiers. *Mutat Res*, **717**, 1-8.

Catto JW, Miah S, Owen HC, et al (2009). Distinct microRNA alterations characterize high- and low-grade bladder cancer. *Cancer Res,* **69**, 8472-81.

Filipowicz W, Bhattacharyya SN, Sonenberg N (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answer sinsight? *Nat Rev Genet*, **9**, 102-14.

Franken NA, Rodermond HM, Stap J, Haveman J, Van Bree C (2006). Clonogenic assay of cells in vitro. *Nat Protoc*, **1**, 2315-9

Garcia JA, Danielpour D (2008). Mammalian target of rapamycin inhibition as a therapeutic strategy in the management of urologic malignancies. *Mol Cancer Ther*, **7**, 1347-54.

Henson BJ, Bhattacharjee S, O'Dee DM, Feingold E, Gollin SM (2009). Decreased expression of miR-125b and miR-100 in oral cancer cells contributes to malignancy. *Genes Chromosomes Cancer*, **48**, 569-82.

Ichimi T, Enokida H, Okuno Y, et al (2009). Identification of novel microRNA targets based on microRNA signatures in bladder cancer. *Int J Cancer*, **125**, 345-52.

Jacobs BL, Lee CT, Montie JE (2010). Bladder cancer in 2010: how far have we come? *CA Cancer J Clin*, **60**, 244-72.

Leite KR, Tomiyama A, Reis ST, et al (2011a). MicroRNA-100 expression is independently related to biochemical recurrence of prostate cancer. *J Urol*, **185**, 1118-22.

Li BH, Zhou JS, Ye F, et al (2011). Reduced miR-100 expression in cervical cancer and precursors and its carcinogenic effect through targeting PLK1 protein. *Eur J Cancer*, **47**, 2166-74.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**, 402-8.

Lu J, Getz G, Miska EA, Alvarez-Saavedra E, et al (2005). MicroRNA expression profiles classify human cancers. *Nature*, **435**, 834-8.

Nagaraja AK, Creighton CJ, Yu Z, et al (2010). A link between mir-100 and FRAP1/mTOR in clear cell ovarian cancer. *Mol Endocrinol*, **24**, 447-63.

Nana-Sinkam SP, Croce CM (2011). MicroRNAs as therapeutic targets in cancer. *Transl Res*, **157**, 216-25.

Necela BM, Carr JM, Asmann YW, Thompson EA (2011). Differential expression of microRNAs in tumors from chronically inflamed or genetic (APC(Min/+)) models of colon cancer. *PLoS One*, **6**, e18501.

Park SJ, Lee TJ, Chang IH. (2011) Role of the mTOR Pathway in the Progression and Recurrence of Bladder Cancer: An Immunohistochemical Tissue Microarray Study. *Korean J Urol*, **52**, 466-73.

Patnaik SK, Kannisto E, Knudsen S, Yendamuri S (2010). Evaluation of microRNA expression profiles that may predict recurrence of localized stage I non-small cell lung cancer after surgical resection. *Cancer Res*, **70**, 36-45.

Saini S, Yamamura S, Majid S, et al (2011). MicroRNA-708 induces apoptosis and suppresses tumorigenicity in renal cancer cells. *Cancer Res*, **71**, 6208-19.

Schedel F, Pries R, Thode B, et al (2011). mTOR inhibitors show promising in vitro activity in bladder cancer and head and neck squamous cell carcinoma. *Oncol Rep*, **25**, 763-8.

Shi W, Alajez NM, Bastianutto C, et al (2010). Significance of Plk1 regulation by miR-100 in human nasopharyngeal cancer. *Int J Cancer*, **126**, 2036-48.

Song T, Xia W, Shao N, et al (2011). Differential miRNA expression profiles in bladder urothelial carcinomas. *Asian Pac J Cancer Prev*, **11**, 905-11.

Tovar V, Alsinet C, Villanueva A, et al (2010). IGF activation in a molecular subclass of hepatocellular carcinoma and pre-clinical efficacy of IGF-1R blockage. *J Hepatol*, **52**, 550-9.

Xing L, Todd NW, Yu L, Fang H, Jiang F (2010). Early detection of squamous cell lung cancer in sputum by a panel of microRNA markers. *Mod Pathol*, **23**, 1157-64.

Yamamoto Y, Matsuyama H, Kawauchi S, et al (2006). Overexpression of polo-like kinase 1 (PLK1) and chromosomal instability in bladder cancer. *Oncology*, **70**, 231-7.