Expression of miR-100 and miR-138 as prognostic biomarkers in Non- Muscle Invasive Bladder Cancer

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Abstract

Background: MicroRNAs alterations are involved in bladder cancer tumorigenesis. The aim of the current study was to evaluate the potential role of miR-100 and miR-138 as prognostic biomarkers in Ta/T1 Non-Muscle Invasive Bladder Cancer (NMIBC).

Methods: We assessed a quantitative RT-PCR analysis of miR-100 and miR-138 in 50 bladder tumor samples (stage Ta/T1) and 4 healthy adjacent tissue. Western blot analysis was used to measure protein expression of FGFR3 and Cyclin D3 in order to know if these targets can be regulated by miR-100 and miR-138 respectively. The statistical analysis included non-parametric tests (Mann–Whitney U and Kruskal–Wallis) and univariate survival analysis by Kaplan-Meier method and the log-rank test.

Results: Low expression of miR-138 characterized recurrent tumors ($p = 0.043$), and higher expression levels were associated with longer recurrence-free survival ($p = 0.012$). However, low miR-100 expression correlated with longer progression-free survival (marginal significance; $p = 0.053$) and cancer-specific overall survival ($p = 0.006$). Additionally, higher levels of miR-100 were associated with negative FGFR3 protein expression ($p = 0.032$) and higher levels of miR-138 were associated with positive Cyclin D3 protein expression ($p = 0.037$).

Conclusion: Our results support miR-138 and miR-100 as prognostic biomarkers in patients with NMIBC.

Keywords: microRNA expression; bladder cancer; stage Ta/T1; prognostic biomarkers.

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Introduction

Non-muscle invasive Ta/T1 bladder cancer (NMIBC) represents about 75% of all urinary bladder tumors; 70% of them are Ta tumors (1, 2). Tumor recurrence and progression are the major events associated with patients in this category. The fact that clinical behaviour of these tumors is frequently unpredictable supports the needed of biomarkers enable to predict both recurrence and/or most importantly progression in NMIBC (2, 3).

Currently, the prognostic factors in bladder cancer are based on clinical and pathological parameters such as tumor grade, tumor stage, multiplicity, size, early recurrence and concomitant carcinoma *in situ,* but unfortunately, these tools are still unable to predict prognosis in some cases (4-6).

Recent data suggest that microRNA profiles can distinguish between different histological types, and separate them in different grade and stage categories (7, 8). Thus, there is strong interest in finding microRNA activity-based biomarkers since they might provide insights regarding which patient may have a better prognosis. Recent studies have reported a number of microRNAs including miR-21, miR-143, miR-155, miR-214, and miR-222, that could potentially serve as risk stratified. However, studies with long-term follow up are needed to verify the potential prognostic significance of these microRNAs (8).

It is well known that microRNAs play a key role in regulating the expression of genes associated with carcinogenesis (9). In bladder cancer, miR-100 can act as tumor suppressor through oncogenes as FGFR3, indicating a very early event in the initiation of bladder tumorigenesis (10). Additionally, previous studies reported that miR-138 plays important roles in several types of tumors including hepatocellular carcinoma, in which cyclin D3 (CCND3) is characterized as a direct target of miR-138 (11). However, whether miR-138 is involved in bladder cancer carcinogenesis remain unexplored.

On the other hand, data on miR-100 and miR-138 in NMIBC is limited, with most references supporting a potential role in prognosis. Likewise, it is still unclear if these two microRNAs could indeed predict of tumor´s recurrence, progression or cancer-specific survival (12-14).

In this study, we report the expressions levels of miR-100 and miR-138 as prognostic biomarkers of recurrence-free, progression-free and cancer-specific survival in a consecutive cohort series of 50 patients with NMIBC and long-term follow up. Our study also reports on the expression of the potential targets of miR-100 and miR-138, at the protein level, that is FGFR3 and Cyclin D3, respectively in NMIBC.

Material and methods

Patients and Samples collection

Fresh tumor tissue of bladder cancer was obtained by transurethral resection of the bladder (TURB) at the Urology Unit, Reina Sofia University Hospital (Cordoba, Spain) in 2005. The Hospital´s ethics committee approved the study, and signed informed consent was obtained by all patients.

The samples were removed in the course of routine surgical procedures and divided into 2 parts: one part was immediately frozen and stored at –80°C until processing, and the second part was fixed in formalin and embedded in paraffin (FFPE). Histological evaluation was carried out using hematoxylin/eosin (H&E) staining. The pathological specimens were re-assessed by one experienced uropathologist (ALB) in order to confirm the diagnosis

The study series consisted of a sequential cohort of 50 (Ta/T1) NMIBC (Table 1). Patients who were diagnosed as carcinomas *in situ* were excluded from this study, in order to delineate a more homogeneous study population. Additional samples consisted of 4 normal urothelial tissues, resected 7 to 10 cm away from the tumor, were also collected. Patients with high grade (stage T1)

bladder carcinoma additionally received intravesical BCG with maintenance following the EAU guidelines (15).

Patient´s follow up was defined as the number of months from the date of the surgical procedure to the date of the latest cystoscopy (or the last visit or death). Recurrence event was defined as reappearance of a tumor after the initial treatment with at least one tumor free cystoscopy interval. Progression event was defined as a shift to any higher stage (T1-T2-T3) in recurrent tumors or the appearance of metastases. Survival time was defined as the period of time between the diagnosis and death. Cancer-related death was that caused by bladder carcinoma. Multifocality was considered positive when two or more papillary lesions in bladder urothelium were present. Regarding tumor size, we pleated tumors as $\leq$ 3cm and > 3cm.

RNA extraction

Total RNA including small RNAs was extracted from pulverized bladder tumor tissue using miRNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. Total RNA concentration was quantified using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA).

MicroRNA expression

MicroRNA expression levels of hsa-miR-100-5p and has-miR-138-5p in tumors and normal bladder tissue were quantitated by RT-qPCR using *EPIK*™ miRNA Select Assays (Bioline, London, UK). Normal bladder tissue and endogenous control U6 snRNA were used to normalize the microRNA expression levels and all experiments were performed in duplicate wells to control technical variance.

100 ng RNA was converted into cDNA using the EPIK™ miRNA RT kit (Bioline, London, UK). The cDNA was diluted 1:10 and 6 µl of cDNA was mixed with 10 µl 2X SensiSMART™ PCR Master Mix, 2 µl PCR

Primer for 3 miRNAs (hsa-miR-100-5p, has-miR-138-5p, hsa-let-7c-5p) and U6 snRNA (Bioline, London, UK) in a total volume of 20 µl. All RT-qPCR experiments were performed using a Bio-Rad CFX connect Real-Time Detection System. Fluorescence and C_t (threshold cycle) values were quantified and converted to raw data with Bio-Rad CFX Manager™ Software (Bio-Rad Laboratories, Inc., Hercules, CA). The melting curve analysis was used to control the specificity of RT-qPCR products.

Fold-change of microRNAs expression was calculated with the $2^{-\Delta\Delta t}$ and normalized with healthy adjacent tissue expression (control). This fold-change was established as biologically significant for a regulation threshold value of +/-4 fold by taking significant p-value 0.01. The classification as "up", "down" or "no change" in regulation refers to fold changes with respect to control.

Protein extraction and Western blotting

MiR-100 targeting of FGFR3 and miR-138 targeting cyclin D3 were investigated using protein expression by Western Blotting method. Qproteome Cell Compartment Kit (Qiagen, Hilden, Germany) was used for protein extraction. The protein content was determined by the method described by Bradford. After that 25 µg of protein from the membrane compartment were electrophoresed in Criterion TGX Stain-Free 4-15% gels (Bio-Rad Laboratories, Inc., Hercules, CA), activated with UV light and finally transferred into nitrocellulose membranes. FGFR-3 Antibody (B-9): sc-13121 and cyclin D3 Antibody (D-7): sc-6283-HRP (Santa Cruz Biotechnology, Inc., CA, USA) were used to incubate nitrocellulose membranes at the dilutions recommended by the manufacturers. The protein levels were normalized by probing the same blots with beta-Actin (C4) Antibody: sc-47778 (Santa Cruz Biotechnology, Inc., CA, USA).

Finally, the detection analysis was carried out using ECL Plus (Amersham Biosciences, Uppsala, Sweden) and the ChemiDoc HRS system and Image Lab Software (BioRad, Hercules CA).

Cyclin D3 protein expression with a band intensity (35KD) higher than control samples was classified as positive. Similarly, FGFR3 protein expression was classified positive when the intensity of both bands (130 and 98 KD approximately) was higher than control. Both biomarkers were considered negative when the bands intensity were near zero (Figure 2).

Statistical analysis

The analysis of clinical data and their association to microRNA expression levels were performed using the statistical software packages SPSS (SPSS version 15; SPSS Inc., Chicago, Illinois) and MedCalc Statistical Software version 17.6 (MedCalc Software bvba, Ostend, Belgium). The relative normalized microRNA expression was characterized by the mean ± SD of at least two independent experiments. T student tests and the non-parametric Mann-Whitney U and Kruskal-Wallis tests were used to evaluate the differences with clinicopathological parameters and protein expression.

Recurrence-free survival (DFS), progression-free survival (PFS) and cancer-specific survival (CSS) were assessed by Kaplan–Meier curves and the differences between the curves were evaluated using the log-rank (Mantel-Cox) test. A significant difference was obtained when a p-value <0.05.

Results

Study Population

Our study series included 50 patients with NMIBC (46 males), with a mean age of 71.5 years (range 44-95). Mean follow-up was 71.9 months (median 73.5 months). Additionally, the mean survival time was 115.8 months (106.6-125.0). Table 1 provides clinicopathological characteristics of the cohort study.

Nineteen (38.0 %) patients were diagnosed as T1/high-grade, 23 (46.0 %) as Ta/low-grade carcinoma, and 8 (16.0 %) as Ta/high-grade urothelial carcinoma.

After initial transurethral resection, recurrence event was noted in 32 (64.0 %) patients; 8 (16.0%) patients had a progression event (2 patients progress from Ta to T1 and 6 from T1 to ≥T2). During follow-up, 10.0 % of the patients died of bladder cancer. A total of 21 (42.0%) and 7 (14.0%) patients had tumor recurrence and progression events up to 5 years of follow up.

Expression of miR-100 and miR-138 in NMIBC: Mean comparisons analysis

Figure 1 shows the expression levels of miR-100 and miR-138 in a total of 50 patients with NMIBC. Comparing Group Means using non-parametric test analysis showed increased levels of miR-138 associated with Ta stage ($p = 0.019$) and low-grade tumors ($p = 0.078$); likewise, decreased expression of miR-138 characterized recurrent tumors ($p = 0.043$).

Next, we determined FGFR3 and Cyclin D3 protein expression in NMIBC using Western blotting method and we investigated whether miR-100 is associated with FGFR3 and if miR-138 is associated with Cyclin D3 protein expression. The analysis revealed that higher levels of miR-100 are associated with negative FGFR3 protein expression ($p = 0.032$). On the other hand, higher levels of miR-138 are associated with positive Cyclin D3 protein expression ($p = 0.037$) (Figure 2, Table 2).

Correlation between microRNA expression levels and patient survival

Kaplan-Meier survival analyses revealed higher expression levels of miR-138 associated with longer recurrence-free survival (log rank test: 8.79; p = 0.012). However, lower miR-100 expression associated with longer progression-free survival and cancer-specific overall survival (marginal significance; log rank test: 5.86; p = 0.053) and (log rank test: 9.78; p = 0.006), respectively (Figure 3).

Discussion

Bladder carcinoma represents a heterogeneous disease group that needs identification and validation of novel molecular biomarkers. NMIBC comprises an important area of clinical research aiming to predict the behaviour of these patients. Many lines of evidence suggest that microRNAs can regulate gene expression acting as emerging regulatory factors, and suggesting their possible roles during tumorigenesis (7, 12). According to microRNA target databases, one microRNA may regulate multiple genes as its targets and similarly, one gene contains potential target sites for numerous microRNAs. More specifically, they can regulate the expression of several proteins (13). In bladder cancer, the use of genome-wide profiling has permitted to identify microRNA alterations, but these studies have some discrepancies due to differences in the sample characteristics and in the methods employed.

In the current study, we evaluated two microRNAs, previously selected from recent literature data as they seem to play key roles in bladder cancer carcinogenic pathways, using fresh-frozen tissue specimen. Some studies suggest that fresh tissue still is considered the most reliable for molecular genetic analysis because they provide a comprehensive analysis of the biopsy (16) .

We used real-time quantitative RT-qPCR, which is a robust methodology with high reliability and reproducibility to explore the expressions of miR-100 and miR-138 in tumor tissues as potential predictors of recurrence, progression and patient survival in NMIBC.

The role of miR-100 in the tumorigenic pathway of NMIBC remain poorly understood, and also its clinical significance has yet to be fully elucidated. Previous data using microarray technology revealed a specific microRNA signature containing 15 miRNAs, of which seven, including miR-100, were upregulated and eight were downregulated in tumor tissue versus healthy tissue (17), nevertheless other authors found that expression levels of miR-100 were distinctly reduced in bladder cancer tissues compared to adjacent normal tissues (18).

However, recently, lower expression of miR-100 in bladder cancer tissues compared to adjacent normal bladder tissues was reported to be correlated with the low grade in NMIBC (10, 19, 20). Indeed, it has been reported that there is a down-regulation of miR-99a, miR-100, miR-101 and miR-145 in NMIBC compared with MIBC (21, 22). Our study showed no statistical association between miR-100 expression levels and grade (LG/HG) nevertheless, it showed a marginal significance with the stage ($p = 0.062$) in NMIBC.

There is an increasing evidence to show that miR-100 plays a tumor suppressor role by targeting oncogenes such as FGFR3 and prevent cancer initiation (23). Several studies have reported a negative correlation between downregulation of miR-100 and FGFR3 expression in bladder cancer (9, 21). Similarly, Catto et al. revealed that higher FGFR3 expression may be increased in the absence of FGFR3 mutation or copy number gain (22). Thus, it is known that activating FGFR3 mutations are thought to be associated with high levels of FGFR3 protein expression (24). Here, we report that FGFR3 protein expression was negative in tumors with higher miR-100 levels, which could suggest that miR-100 regulate the level of FGFR3 protein.

Concerning clinical significance, there are controversial results with some studies showing that miR-100 downregulation is associated with unfavourable prognosis (8). Nevertheless, other studies, which are in line with our result, indicate that miR-100 downregulation is associated with a better outcome (18, 22). Thus, our results have shown shorter progression-free survival and cancer-specific survival of NMIBC patients with higher miR-100 expression.

MiR-138 has recently emerged as a promising therapeutic target for several cancers such as cervical cancer, osteosarcoma, non-small-cell lung cancer, ovarian cancer, and gallbladder carcinoma (25). Previous studies have reported that miR-138 is frequently downregulated in colorectal cancer and is associated with an advanced clinical stage, lymph node metastasis and poor overall survival (26). However, to our best knowledge, this is one of the first studies to determine the potential clinical significance of miR-138 in NMIBC. Interestingly, our data illustrate that miR-l38 overexpression reached statistical significance with Ta stage ($p = 0.019$**)** and a marginal significance with the low grade ($p = 0.078$).

Reportedly miR-138 acts as a tumor suppressor by targeting many target genes (25). In bladder cancer, it is known that miR-138 overexpression inhibits bladder cancer cells by targeting ZEB2 and contributes to cell proliferation and invasion by targeting Survivin (25, 27, 28). On the other hand, the observation of miR-138 downregulation in bladder cancer is consistent with a similar observation in head and neck cancer and thyroid cancer, in which downregulation of miR-138 has been associated with enhanced telomerase reverse transcriptase (TERT) expression. Moreover, recent findings suggest that miR-138 could play an important role in the development of cisplatin resistance in non-small cell lung cancer, a key drug frequently used in muscle-invasive bladder cancer (29-32).

TargetScan and miRanda predictions indicate that CCND3, important regulatory factor on G1/S phase of cell cycle, could be a potential target of miR-138 which has been corroborated in hepatocellular carcinoma, (11). On the other hand, miR-138 decreased cell proliferation, and tumor

growth and increased cisplatin sensitivity through targeting multiple genes, including G protein-coupled receptor kinase interacting ArfGAP 1 (GIT1), semaphorin 4C, cyclin D3, Glucose-regulated protein 124 (GRP124), enhancer of zeste homolog 2 and pyruvate dehydrogenase kinase 1 (33). To clarify the relationship between miR-138-5p and Cyclin D3 in NMIBC we used western blot analysis. Interestingly, although we expected to find higher levels of miR-138 associated with negative Cyclin D3 protein expression our results indicated that higher miR-138 expression is associated with positive Cyclin D3 protein expression in NMIBC. Therefore, bioinformatics prediction combined with luciferase reporter assay are needed to verify whether CCND3 is a potential target gene of miR-138 in bladder cancer, but this analysis is out of the aim of the current study.

Finally, the survival analysis showed that higher expression levels of miR-138 were associated with better recurrence-free survival indicating that miR-138 could be a good biomarker for NMIBC patients. These results are in line with other studies that show that bladder cancer patients with lower miR-138-5p levels have worse 5 years overall survival and recurrence-free survival rates (34).

The main limitation of this study is the sample size, but however, our cases present a long follow up (median of 120 months), which add value since it can provide a better significance to our observations concerning the prognostic value of these microRNAs. Other limitations include the lack of urothelial CIS cases and the analysis of inverted growth features in our series. These were not evaluated to avoid to reduce potential confounding factors in a small series.

In summary, we identified biomarkers miR-100 as a potential predictor of tumor progression and cancer-specific survival in our cohort series of 50 consecutive NMIBC patients with long-term follow up (mean 71.92 months). Also, we have observed that miR-138 defines recurrence-free survival in our cohort in NMIBC. Therefore, both biomarkers show complimentary predictive ability. In conclusion, our study suggests a potential clinical utility of a panel of two microRNAs (miR-100 and miR-138) as predictors of aggressive behaviour in NMIBC tumors treated by transurethral resection and intravesical BCG immunotherapy.

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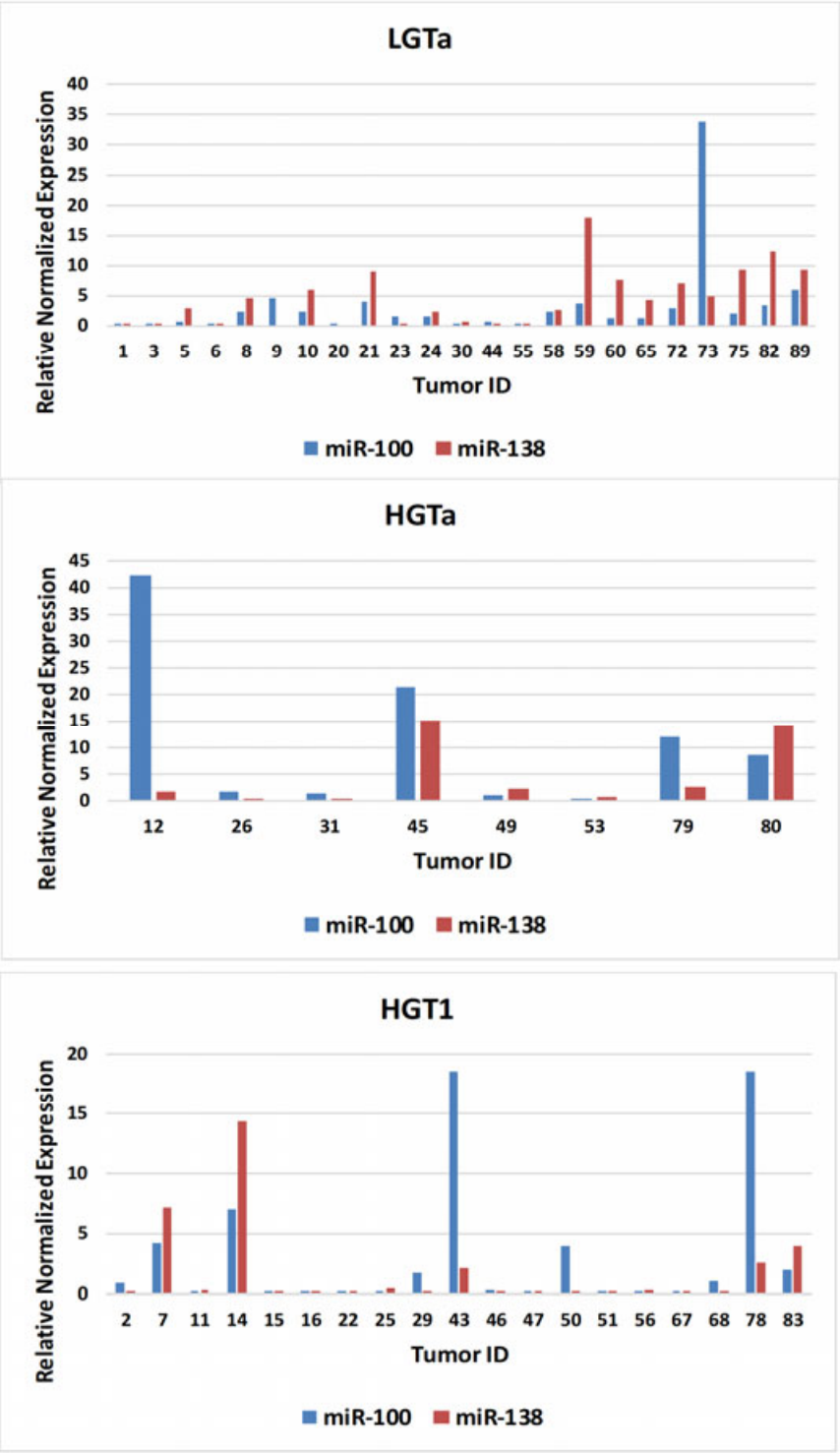

Figure 1. Relative Normalized Expression profile of miR-100 and miR-138 in LGTa, HGTa and HGT1 tumors.

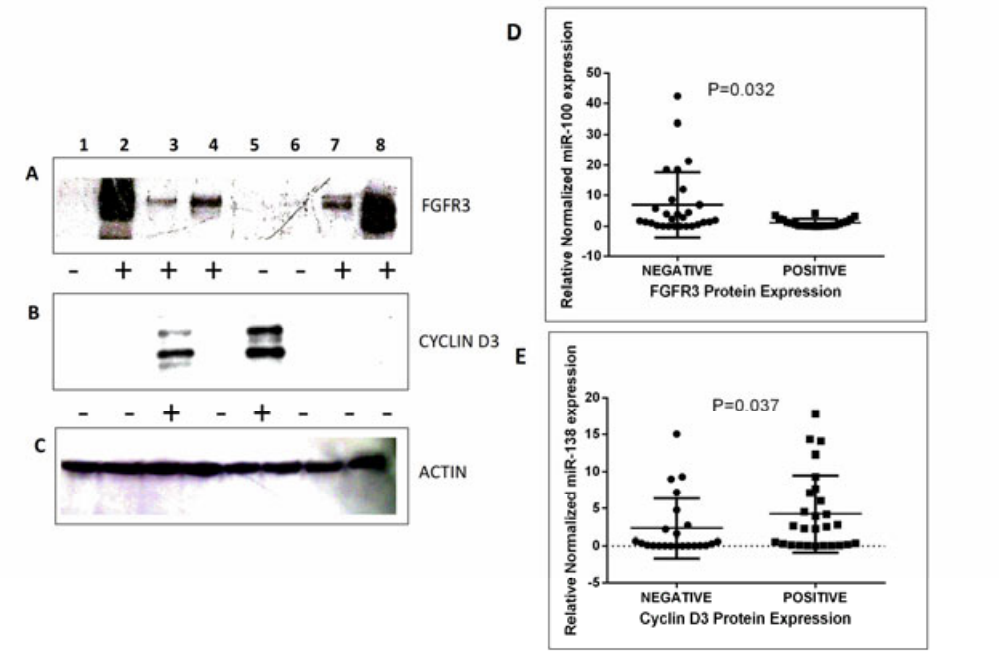

Figure 2. Western blot analysis of FGFR3 (A), Cyclin D3 (B) and Actin (C) as a loading control using fresh frozen bladder tumors tissue samples. Figure 2A: Line 1 non-neoplastic urinary bladder Anti-FGFR3 antibody negative. Lines 2,3,4,7 and 8 Anti-FGFR3 antibody positive. Figure 2B: Line 1 non-neoplastic urinary bladder Anti-Cyclin D3 antibody negative. Lines 3 and 5 Anti-Cyclin D3 antibody positive. Western blot analysis showed bands at 35 kD consistent with the Cyclin D3 protein and analysis of FGFR3 showed bands forming a smear of the glycosylated form (120-130 kD). Association of the relative normalized expressions of miR-100 with FGFR3 (D) and of miR-138 with Cyclin D3 protein expression (E).

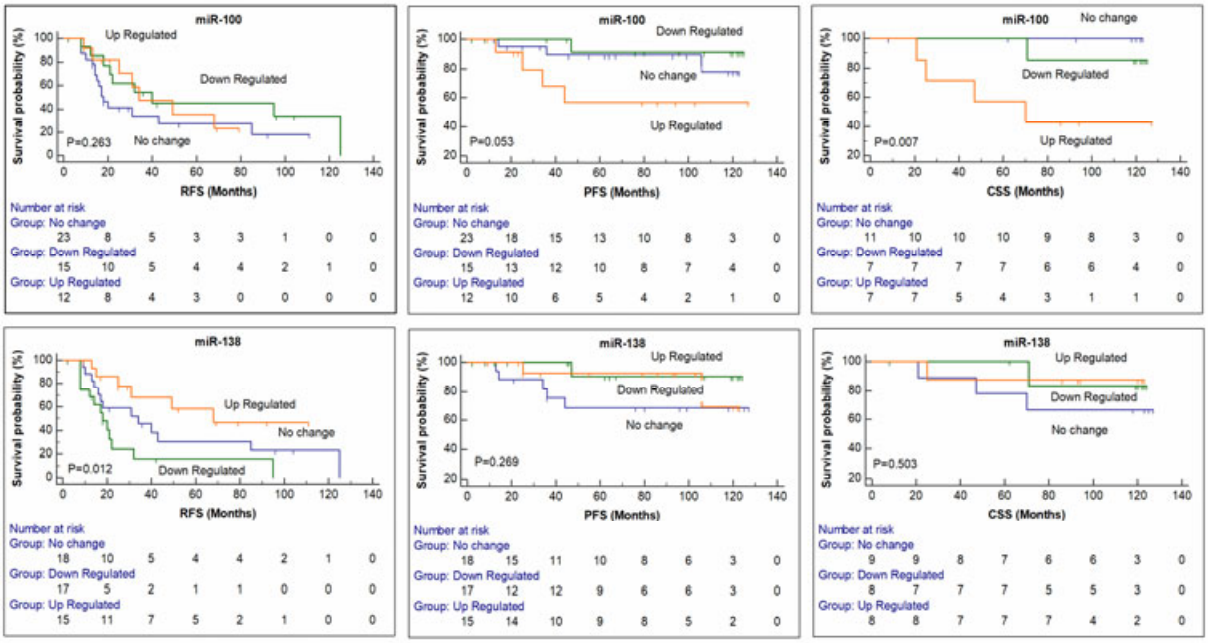

Figure 3. Kaplan-Meier plots for Recurrence-free survival (RFS), Progression-free survival (PFS) and cancer especific (CSS) survival curves in NMIBC patients according to relative normalize expressions of miR-100 and miR-138.

Table 1. Clinicopathological characteristics of patients.

Patients data	**N (%)**
Mean age±SD**, years (range)**	71.5±11.8 (44-95) (Median, 72.5 years)
Mean follow-up±SD**, months**	71.9±42.5 (Median, 73.50 months)
Mean follow-up dead±SD, **months**	46.8±23.8 (Median, 47.00, months)
Mean follow-up alive±SD, **months**	108.2±28.9 (Median, 120 months)
Gender	
Female	4 (8.0)
Male	46 (92.0)
Tumor grade (WHO 2004/2016)	
Low	23 (46.0)
High	27 (54.0)
T category	
Ta	31 (62.0)
T1	19 (38.0)
Multifocality	
Single	20 (40.0)
Multiple	30 (60.0)
Tumour size	
< 3 cm	22 (44.0)
> 3cm	28 (56.0)
Recurrence event	

No	18 (36.0)
Yes	**32 (64.0)**
≤12 months 13-60 months ≥ 61 months	7 (14.0) 21 (42.0) 4 (8.0)
Progression event	
No	42 (84.0)
Yes	**8 (16.0)**
T1 to ≥ T2 Ta to T1 ≤12 months 13-60 months ≥ 61 months	6 (12.0) 2 (4.0) 0 (0.0) 7 (14.0) 1 (2.0)
Survival	
Dead Bladder Cancer	5 (10.0)
Dead other causes	25 (50.0)
Alive	20 (40.0)

Table 2. Mean comparison of the relative expression for miR-100 or miR-138 with clinicopathological parameters in study.

	miR-100	***p-value****	***miR-138***	***p-value****
Gender		0.272		0.142
Female	3.6±3.3		7.0±6.0	
Male	4.5±8.8		3.0±4.5	
Tumour grade (WHO 2004/2016)		0.553		**0.078**
Low	3.3±6.8		4.4±4.7	
High	5.4±9.7		2.5±4.6	
T category		**0.062**		**0.019**
Ta	5.3±9.8		4.4±5.1	
T1	3.1±5.7		1.7±3.6	
Multifocality		0.937		0.440
Single	6.3±11.8		3.9±4.9	
Multiple	3.2±5.2		3.0±4.6	
Tumour size		0.784		0.696
< 3 cm	4.2±8.1		3.6±5.0	
> 3cm	4.6±8.9		3.2±4.5	
Recurrence event to 10 years		0.642		**0.043**
No	5.5±8.9		5.8±6.3	
Yes	3.9±8.3		2.7±2.8	
Progression event to 10 years		0.460**		0.653**
No	4.1±8.7		3.5±5.1	

T1 to ≥ T2	7.3±8.8		3.1±2.3	
Ta to T1	2.9±2.4		2.1±2.9	
Survival to 10 years		0.191#		0.613#
Alive or Dead other causes	3.9±8.4		3.5±4.9	
Dead Bladder Cancer	9.2±8.7		2.3±2.9	
FGFR3 protein expression		**0.032**		
Negative	6.9±10.7			
Positive	1.2±1.3			
Cyclin D3 protein expression				**0.037**
Negative			2.3±4.0	
Positive			4.3±5.2	

*U de Mann Whitney test

** Kruskal-Wallis test

t student test