PROGNOSTIC VALUE OF microRNA EXPRESSION PATTERN IN UPPER TRACT UROTHELIAL CARCINOMA

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: doi/10.1111/bju.12551

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Conflict of interest: The authors declare that they have no competing interests.

Word count of the text: 3237 (excluding abstract)

Word count of the abstract: 246

ABSTRACT

Objective To examine the microRNA (miRNA) expression pattern in tumour samples of progressing and non-progressing upper tract urothelial carcinoma (UTUC) patients in order to identify putative miRNAs that may be used as prognostic markers.

Subjects and methods Multicenter, retrospective study of formalin-fixed paraffin embedded tissue samples from 150 UTUC patients who underwent radical nephroureterectomy. Global miRNA expression patterns were analyzed in 18 selected samples from UTUC patients using TaqMan arrays. Differential expression of five key miRNAs was validated by quantitative PCR in an independent cohort of 132 samples from UTUC patients. Tumour progression and cancer-specific survival predicting models, including miRNA expression patterns, were developed by Cox regression analysis.

Results Twenty-six miRNAs were found to be aberrantly expressed between progressing and non-progressing UTUC patients and five of these were selected for

subsequent studies. The regression analysis identified tumour stage and miR-31 and miR-149 expression as independently associated with tumour progression and tumour stage and miR-149 expression as independently associated with cancer-specific survival.

The risk scores (RS) derived from these miRNAs models were able to discriminate two groups with a highly significant different probability of tumour progression (HR 4,78; p<0.001) and death (HR 2.76; p=0.0036).

Conclusion There is a differential miRNA expression pattern between progressing and non-progressing UTUC patients. Identification of new miRNAs associated with a high probability of tumour recurrence and cancer-specific survival in UTUC patients and their combination in a robust, easy-to-use and reliable algorithm may contribute to tailor treatment and surveillance strategies in these patients.

KEY-WORDS (MeSH)

microRNAs; prognosis; real time PCR; upper tract urothelial carcinoma

INTRODUCTION

Radical nephroureterectomy (RNU) is the gold-standard treatment for localized upper tract urothelial carcinoma (UTUC) [1]. There are only a few established prognostic factors associated with tumour progression and survival, notably pathological stage and tumour grade, but these are insufficient to predict the individual outcome of UTUC patients [2]. Predictive tools such as nomograms have been proposed after RNU but they are still lacking high accuracy [3]. Thus, more accurate knowledge regarding the biological behaviour of tumours would allow tailored treatment schedules to be offered to patients, in an attempt to increase survival and decrease morbidity.

The rapid advance in the understanding of the molecular biology of carcinogenetic processes has lead to the appearance of promising new cancer biomarkers such as microRNAs (miRNAs). miRNAs are a class of small non-coding RNAs that regulate various biological processes post-transcriptionally and are dysregulated in most cancer types [4-7]. However, to our knowledge, the complete miRNA profiling of UTUC patients has not yet been explored. Unlike mRNA, miRNAs have been shown to be unusually well-preserved in a range of specimens, including formalin-fixed paraffin embedded tissue samples [8]. This stability offers a distinct advantage of miRNA over mRNA as the analyte in the clinical setting, and has led to a considerable interest in the development of miRNAs as biomarkers for molecular diagnostic, prognostic and therapeutic applications.

Here, we aimed to examine, for the first time, the miRNA expression profiles of progression and non-progression UTUC patients in order to identify putative miRNAs that may be used as prognostic markers.

PATIENTS AND METHODS

Patients

A retrospective study in which a total of 150 patients (mean age 70 yr, range 45-101 yr; 34 females, 116 males) with UTUC who underwent nephroureterectomy in three different centers (Hospital Clinic of Barcelona-Spain, Pitié Salpétrière Hôspital of Paris-France and Claude Huriez Hôspital of Lille-France) between 1990 and 2004 were included. The only exclusion criterion was the lack of tissue from the archive blocks. Pathological characteristics of the UTUC patients are shown in table 1. Tumours were graded and classified according to the WHO's [9] and the TNM's classification of the International Union Against Cancer [10]. Tissue samples were obtained under institutional review board-approved protocol.

The median follow-up of the studied population was 46 months (range 3-213 mo). All patients were followed-up postoperatively in three-month intervals for the first year, in 6-month intervals for the next two years, and annually thereafter. Tumour was considered to be in progression when distant metastasis or pathological nodes were developed during the follow-up.

Tissue specimens and RNA isolation

Upon obtainment the tissue was fixed in 10% formalin within 24 h and subsequent embedded in paraffin. A slide of each specimen was stained with haematoxylin-eosin to determine the presence of tumour cells. Only those specimens with a minimum of 75% of tumour cells were considered for further analysis. Total RNA was isolated from specimens (80-μm) using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion INC) according to the manufacturer's protocol. Total RNA was quantified by spectrophotometric analysis at 260 nm (NanoDrop Technologies, Wilmington, DE, USA).

Global screening phase

A flowchart of the entire study in shown in Figure 1. Global miRNA profiling of 18 randomly selected UTUC cases from Hospital Clinic of Barcelona, nine progressing (pTa=1, pT1=1, pT2=1, pT3=4, pT4=2; G1=7, G2=1, G3=1; mean progression= 17.2 months) and nine non-progressing (pTa=1, pT1=6, pT2=1, pT3=1; G2=6, G3=3; mean follow-up= 94 months), was performed using TaqMan® Array Human MicroRNA A+B Cards Set v2.0 (TA) that contain 754 unique assays specific to human miRNAs and four control assays in each card (Applied Biosystems, Foster City, USA, hereafter referred to as AB) according to manufacturer's instructions. Briefly, miRNAs were reverse transcribed with the TaqMan MicroRNA Reverse Transcription kit (AB) using 500 ng of total RNA following manufacturer's instructions (Megaplex RT primers for Human Pool A and B, AB). After reverse transcription, 2.5 µl of cDNA was preamplified with Megaplex PreAmp primers following manufacturer's instructions (AB), except that the final volume of the reaction was 25 µl instead of 50 µl. Then, an aliquot of 1 µl of preamplified cDNA was applied to verify the actual amount of RNU48 using the specific primer/probe set (AB) via quantitative PCR (qPCR). Standard reaction and amplification conditions were used (final volume of qPCR reaction of 10 µl). Those samples that provided cycle quantification (Cq) value for the RNU48 lower than 17-18 were diluted with water to ensure a homogeneous amount of cDNA in all samples and the correct quantification of the miRNAs. Afterwards, miRNAs were analyzed in TA using an ABI7900HT instrument.

Technical validation of differentially expressed miRNAs

To select miRNAs for technical validation using a different methodology, we used the false discovery rate (FDR) ≤10% and absolute fold change (FC) ≥ 2 between distinct groups in TaqMan® Human MicroRNA Array (n=21). Then, we checked whether these differentially expressed miRNAs belonged to the same miRNA cluster and only one miRNA from each was then selected for further

validations (For example clusters hsa-miR-200a, hsa-miR-200b, hsa-miR-200a*, hsa-miR-200b* or hsa-miR-141, hsa-miR-200c, hsa-miR-141* containing several differentially expressed miRNAs from our list). Finally, a total of eight differentially expressed miRNAs obtained in TA were selected for validation in the 18 previously analyzed samples, using miRCURY LNA Universal RT microRNA PCR kit (Exiqon, Vedbaek, Denmark).

RT-qPCR reactions were performed according to the manufacturers' instructions (Exiqon, Vedbaek, Denmark). Briefly, cDNA was synthesized using a poly(T) primer and was amplified with locked nucleic acid (LNA) primers and SYBR Green master mix. Specific LNA PCR primer sets used were hsa-miR-200a, hsa-miR-31, hsa-miR-493, hsa-miR-99a, hsa-miR-378*, hsa-miR-181a-2*, hsa-miR-149 and hsa-miR-141. hsa-miR-218 was used as an endogenous control. PCR reactions were carried out using standard conditions in an ABI7900HT instrument. At the end of the PCR cycles, melting curve analyses were performed.

Classifier discovery phase

A total of five key miRNAs (miR-31, miR-493, miR-99a, miR-181a-2* and miR-149) were selected for validation in an independent series of 132 tissue samples; 56 from Claude Huriez Hospital of Lille, 16 from Pitié Salpétrière Hospital of Paris and 60 from Hospital Clinic of Barcelona. **miR-493, miR-99a and miR-181a-2* were selected because we found that they were differentially expressed ($p<0.05$) between both groups via two different techniques in the same cohort of patients (Table 2). Moreover, we also decided to further analyze miR-31 and miR-149 expression in the independent cohort because, although their expression was not statistically significant between both groups in the technical validation step, they had an absolute FC ≥2 and their differential expression was in the same direction using both RT-qPCR based techniques. It should be taken into account that the initial cohort of patients was small (n=18), so statistically significant differences should**

be analysed with care and furthermore, we were searching for biomarkers predictors of tumour progression and cancer-specific survival, and it has been previously reported that not significance but discrimination is important for a biomarker [11].

RT-qPCR reactions were performed using miRCURY LNA kit as described above

Data analysis

TaqMan® Human MicroRNA Array: RT-qPCR data was processed with SDS 2.4 and Enterprise software packages (AB). An automatic threshold and baseline was used for all the miRNAs to record the Cq value. Data normalization was carried out using a global mean normalization method [12]. Subsequently, miRNAs with expression levels correlating to the global mean Cq values were identified and miR-218 was selected by using GeNorm as reference miRNA [13].

Those miRNAs with Cq values above 35 in at least 45% of samples were filtered out, giving a total of 409 valid miRNAs. Relative expression levels of target miRNAs within a sample was expressed as ΔCq ($\Delta Cq = Cq_{miR\text{-}218} - Cq_{target\ miRNA}$). miRNAs with Cq values above 35 were considered as lowly expressed, and their ΔCq were imputed to minimum ΔCq value for that miRNA. Fold change values were generated from the median expression of the miRNAs from the TaqMan® Human MicroRNA Array in the groups compared. Differences in miRNA expression levels between progressing and non-progressing patients were explored using the Student's *t* test. Significance was defined as False Discovery Rate (FDR) values of less than 10%. R-software was used for all calculations and to construct heatmap.

microRNA LNA™ real-time RT-qPCR: No miRNAs with Cq values above 35 were found when using LNA primers. Samples with a miR-218 Cq value higher than 30 were considered to have low RNA quality and were excluded from the analysis. ΔCq values were calculated as described above.

Statistical analysis

Univariate Cox regression analysis was performed on each covariate to examine its influence on tumour progression and cancer–specific survival. Thereafter, a multivariate forward stepwise Cox regression analysis was performed. Statistical significance was established at α-value of 0.05. SPSS 12.0 software was used for statistical analysis.

After establishing the multivariate model, a risk score (RS) for the miRNAs of the model was calculated for each patient according to the general form RS= exp $\Sigma\beta ixis$, where $i = 1, \ldots, k$ index variables, βi represents the coefficient for each variable estimated from the Cox regression model, and *xis* the corresponding value for each variable in a given patient. RS was subjected to a ROC analysis in order to choose the most appropriate threshold for predicting tumour progression and cancer-specific survival. Thereafter, Kaplan-Meier curves were generated using the selected cut-off point and compared according to the log-rank test. Since progression and time of death was not available for three patients, survival analyses were performed using the 147 available patients.

Pathway enrichment analysis

DIANA-mirPath tool [14], using TargetScan as the target prediction algorithm, was used to identify targets of the key miRNAs, and subsequent target enrichment analysis was performed in order to discover possible canonical altered pathways.

RESULTS

Global screening phase

Overall, analysis of TaqMan® Human MicroRNA Array-derived expression data from nine progression and nine non-progression cases resulted in the identification of 26 miRNAs with a FDR of less than 10%; 20 downregulated and six upregulated miRNAs

in deceased with respect to live patients. Heat map based on differentially expressed miRNAs shows a distinction between progression and non-progression group (Figure 2).

Technical validation of differentially expressed miRNAs

To assure the consistency in the experimental procedures followed in the screening phase, we used a different approach, based on LNA^{TM} PCR primer sets, to quantify eight selected miRNAs in the same 18 samples evaluated using the TaqMan® Human MicroRNA Array. Even though there are several methodological differences between both platforms, six of the eight miRNAs tested maintained the same fold change direction when analyzed with LNA^{TM} PCR primer sets and in three of them (miR-181a-2*, miR-493 and miR-99a), these differences were statistically significant ($p<0.05$) (Table 2).

miRNAs associated with tumour progression and cancer-specific survival

In order to identify miRNAs correlated with patient's progression and survival, expression levels of five key miRNA, were analyzed by RT-qPCR in an independent cohort of 132 UTUC tissue samples. During the follow-up period of these 132 patients, 39 (26%) developed tumour progression and 37 (24.7%) died due to UTUC. Five-year tumour progression and cancer-specific survival of the series were 71.67% and 70.13%, respectively. The mean time to tumour progression and death were 15.91 and 30.81 months, respectively.

To verify whether these five selected miRNAs were independent prognostic factors of patient's progression and survival, the miRNAs and the clinical variables in all 132 patients were analyzed by Cox regression model. First, the univariate analysis revealed significant predictors of tumour progression and cancer-specific survival (Table 3). Then, the multivariate regression analysis demonstrated that pathological tumour stage and expression of miR-31 and miR-149 were independent prognostic factors of tumour

progression (HR 2.46; p<0.001, HR 0.88; p<0.001 and HR 0.78; p=0.006, respectively) and pathological tumour stage and miR-149 expression were independent prognostic factors of cancer-specific survival (HR 1.79; p=0.0001 and HR 0.82; p=0.0183, respectively).

The RS for tumour progression was calculated for each patient according to a mathematical algorithm containing miR-31 and miR-149 expression values. The median value of this RS was 0.574 (range 0.043-10.98). Thereafter, a ROC analysis of the model allowed selecting a cut-off value of 0.86 to classify patients into a high-risk group of tumour progression (27%) and low-risk group of tumour progression (73%). Figure 3A depicts Kaplan-Meier curves generated using the selected cut-off point. As shown, RS generated using miRNA expression values was able to discriminate two groups with a highly significant different probability of tumour progression (HR 4.78; p<0.001).

In parallel, the RS for cancer-specific survival was calculated using miR-149 expression values (RS median value=0.784; range 0.188-2.431). The subsequent ROC analysis allowed selecting a cut-off value of 0.93 to classify patients into a high-risk group of cancer-specific survival (35%) and low-risk group of cancer-specific survival (65%). The RS generated was able to discriminate two groups with a significantly different probability of cancer-specific survival (HR 2.76; p=0.0036) (Figure 3B).

DISCUSSION

Around 30% of UTUC patients analyzed in this study died due to their tumour after 5-years follow-up. Pathological stage and histological grade are the established prognostic factors for UTUC but they are insufficient to predict individual tumour behaviour. Thus, it would be of interest to find more reliable and individualized prognostic markers. To this end, different molecular markers have been previously

evaluated in samples from UTUC patients by using immunohistochemistry (IHC) [15-21] or *in situ* hybridization [22], however, none of them have been incorporated into the clinical setting. We have also previously analyzed gene expression patterns of several genes in UTUC samples but we were not able to identify prognostic factors of UTUC based on the genes analyzed [15].

miRNAs have been described as novel prognostic molecules involved in several tumours [4-7]. However, to our knowledge, miRNA expression profiles of progression and non-progression UTUC patients had not been explored as yet. In the current study, we first investigated global miRNA expression patterns in tissue samples from a reduced cohort of UTUC patients. Here we identified a list of 26 miRNAs differentially expressed between progressing and non-progressing UTUC patients. However, we were aware that although global miRNA expression profiling of UTUC samples provides miRNAs implied in UTUC progression, it represents early data that needs further validation. To this end, we first selected eight differentially expressed miRNAs to be validated in the same cohort using a different real time PCR-based approach. Five of the eight miRNAs were technically validated indicating that although both approaches used in this study are real time PCR-based, methodological differences between both qPCR platforms such as the priming system for reverse transcription, the employment of a preliminary cDNA preamplification step and the chemistry used in the qPCR reaction could result in some discrepancies. In fact, the partial validation of global profiling studies by RT-qPCR as well as differences in the magnitude of change have been previously reported [23].

Finally, in order to identify miRNAs that correlate with tumour progression and shorter survival, we tested these five miRNAs in an independent, larger, multicentre cohort of UTUC patients. As shown, tumour stage and miR-31 and miR-149 expression independently predict tumour progression and moreover, tumour stage and miR-149 expression independently predicts cancer–specific survival. As a result, the RS derived

from miRNAs in our multivariate model was able to discriminate two groups with a highly significant different probability of tumour progression (HR=4.78; p<0.001) and cancer–specific survival (HR=2.76; p=0.0036). Thus, a model composed of miR-31 and miR-149 provides a robust, easy-to-use system to identify a subgroup of patients with a higher probability of tumour progression, while expression of miR-149 is able to identify a subgroup of patients with shorter survival. Thus, the analysis of these two miRNAs in UTUC tissue samples refines the currently used clinico-pathological-based approach by adding the analysis of a limited number of genetic markers which could be very useful for making decisions in clinical practice.

Regarding the genetic markers included in our algorithm, miR-149 has been found to be dysregulated in many tumours including clear renal cell carcinoma, squamous cell carcinoma of the tongue, prostate cancer, glioblastoma and astrocytoma. In addition, it has been described to serve as a diagnostic and prognostic marker for bladder and colorectal cancer [24,25]. On the other hand, miR-31 expression has been found altered not only in bladder cancer but also in prostate, gastric, breast and serous ovarian cancer [26], but functional roles for miRNA-31 have yet to be defined.

Several possible pathways were predicted to be modified by the key miRNAs miR-31 and miR-149. The statistically significant altered pathways in tumour progression and cancer-specific survival are shown in Table 4. Notably, some of these altered pathways predicted by these two miRNAs have been previously associated with other cancers, specially hematologic and breast neoplasms [27,28].

Regarding the application of miRNAs in the clinical setting, it is of interest to consider that miRNAs have some methodological advantages over gene expression studies. First, it is harder to obtain high quality long-chain mRNA from tissue samples. On the other hand, short mature miRNAs are more stable against nuclease degradation due to their smaller size and actually, the isolation of high quality miRNA from FFPE blocks has already been reported [8], suggesting that miRNAs may escape the chemical

degradation induced by formalin fixation. Also, the average copy number of an individual miRNA species has been estimated at approximately 500 per cell, which may be higher than the average expression of mRNA species [29]. This implies that less total RNA is required for a miRNA than for an mRNA expression experiment, which is an important advantage when working with clinical samples**.**

The strength of this study relies on the fact that we have used a nontargeted, exploratory approach to select the candidate miRNAs. Furthermore, we have used archival FFPE samples to obtain miRNA expression patterns allowing an easy translation of the results obtained to clinical practice. Finally, a multicenter cohort with prospective data collection and long term follow-up is analyzed in the present study, which eliminates the limitations of a single population study. We are aware, however, of some limitations of the study. First, we chose a group of eight miRNAs to validate from the initial study where 26 differentially expressed miRNAs were shown. It remains possible that we may have ruled out some miRNAs highly predictors of tumour progression and cancer-specific survival. Second, although we have tried to include a substantial number of patients from three different centres, the total number of patients analyzed can still be considered as low. Moreover, because of our interest in identifying robust markers, all available patients were used to discover prognostic miRNAs, thus preventing an independent validation. In that sense, although the data reported warrants further prospective evaluation in carefully and specifically designed studies, our study may contribute to the identification of a reliable prognostic system for UTUC patients.

In conclusion, our results demonstrate that there is a differential miRNA expression pattern between non-progressing and progressing UTUC patients. We also show that an algorithm that combines miR-31 and miR-149 expression is able discriminate two groups associated with different probability of tumour progression. Furthermore, miR-149 expression was able to distinguish two groups with different cancer-specific

survival. Although independent validation of the data is necessary, identification of new miRNAs associated with a high probability of tumour recurrence and cancer-specific survival in UTUC patients and its combination in a robust, easy-to-use and reliable algorithm may contribute to tailor treatment and surveillance strategies in these patients.

ACKNOWLEDGEMENTS

We thank Helena Kruyer for the English correction of the manuscript. This work was supported by grants from the Spanish Urological Association (FIU 2010 to LI). MI-T has a Research Support Staff Grant from Inst. Salud Carlos III (CA07/00221).

REFERENCES

1 **Roupret M, Zigeuner R, Palou J et al.** European guidelines for the diagnosis and management of upper urinary tract urothelial cell carcinomas: 2011 update. *Eur Urol* 2011; 59:584-94

2 **Huben RP, Mounzer AM, Murphy GP.** Tumor grade and stage as prognostic variables in upper tract urothelial tumors. *Cancer* 1988; 62:2016-20

3 **Roupret M, Hupertan V, Seisen T et al.** Prediction of cancer-specific survival after radical nephroureterectomy for upper tract urothelial carcinoma: development of an optimized post-operative nomogram using decision curve analysis. *J Urol* 2012; 189:1662-9

4 **Lin T, Dong W, Huang J et al.** MicroRNA-143 as a tumor suppressor for bladder cancer. *J Urol* 2009; 181:1372-80

5 **Vogt M, Munding J, Gruner M et al.** Frequent concomitant inactivation of miR-34a and miR-34b/c by CpG methylation in colorectal, pancreatic, mammary, ovarian, urothelial, and renal cell carcinomas and soft tissue sarcomas. *Virchows Arch* 2011; 458:313-22

6 **Yamada Y, Enokida H, Kojima S et al.** MiR-96 and miR-183 detection in urine serve as potential tumor markers of urothelial carcinoma: correlation with stage and grade, and comparison with urinary cytology. *Cancer Sci* 2011; 102:522-9

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

8 **Hui AB, Shi W, Boutros PC et al.** Robust global micro-RNA profiling with formalin-fixed paraffin-embedded breast cancer tissues. *Lab Invest* 2009; 89:597-606

9 **Lopez-Beltran A, Sauter G, Gasser T et al.** World Health Organization. Classification of Tumours. Pathology and Genetics. Tumours of the Urinary System and Male Genital Organs. Lyon: IARC Press, 2004

10 **Sobin LH, Wittekind Ch.** TNM Classification of Malignant Tumours, 6th ed. New York: Wiley, 2002

11 **Bossuyt PM, Reitsma JB, Bruns DE et al.** Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. *Ann Clin Biochem* 2003; 40:357-63

12 **Mestdagh P, Van Vlierberghe P, De Weer A et al.** A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol* 2009; 10:R64

13 **Vandesompele J, De Preter K, Pattyn F et al.** Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; 3:RESEARCH0034

14 **Papadopoulos GL, Alexiou P, Maragkakis M, Reczko M, Hatzigeorgiou AG.** DIANA-mirPath: Integrating human and mouse microRNAs in pathways. *Bioinformatics* 2009; 25:1991-3

15 **Izquierdo L, Truan D, Petit A, Gutierrez R, Mallofre C, Alcaraz A.** Adhesion molecules alpha, beta and gamma-catenin as prognostic factors of tumour progression in upper urinary tract urothelial tumours: the role of AKT-P/GSK-3beta/beta-catenin pathway. *BJU Int* 2009; 104:100-6

16 **Nakanishi K, Kawai T, Torikata C, Aurues T, Ikeda T.** E-cadherin expression in upper-urinary-tract carcinoma. *Int J Cancer* 1997; 74:446-9

17 **Nakanishi K, Hiroi S, Tominaga S et al.** Expression of hypoxia-inducible factor-1alpha protein predicts survival in patients with transitional cell carcinoma of the upper urinary tract. *Clin Cancer Res* 2005; 11:2583-90

18 **Kashibuchi K, Tomita K, Schalken JA et al.** The prognostic value of E-cadherin, alpha-, beta-, and gamma-catenin in urothelial cancer of the upper urinary tract. *Eur Urol* 2006; 49:839-45

19 **Izquierdo L, Truan D, Mengual L, Mallofre C, Alcaraz A.** HER-2/AKT expression in upper urinary tract urothelial carcinoma: prognostic implications. *Anticancer Res* 2010; 30:2439-45

20 **Kamai T, Takagi K, Asami H, Ito Y, Arai K, Yoshida KI.** Prognostic significance of p27Kip1 and Ki-67 expression in carcinoma of the renal pelvis and ureter. *BJU Int* 2000; 86:14-9

21 **Eltz S, Comperat E, Cussenot O, Roupret M.** Molecular and histological markers in urothelial carcinomas of the upper urinary tract. *BJU Int* 2008; 102:532-5

22 **Nakanishi K, Kawai T, Hiroi S et al.** Expression of telomerase mRNA component (hTR) in transitional cell carcinoma of the upper urinary tract. *Cancer* 1999; 86:2109-16

23 **Mengual L, Burset M, Ars E et al.** Partially degraded RNA from bladder washing is a suitable sample for studying gene expression profiles in bladder cancer. *Eur Urol* 2006; 50:1347-55

449 24 **Mengual L, Lozano JJ, Ingelmo-Torres M, Gazquez C, Ribal MJ,**
450 **Alcaraz A.** Using microRNA profiling in urine samples to develop a non-invasive
451 test for bladder cancer. *Int J Cancer* 2013;

452 25 **Wang F, Ma YL, Zhang P et al.** SP1 mediates the link between
453 methylation of the tumour suppressor miR-149 and outcome in colorectal cancer.
454 *J Pathol* 2012; 229:12-24

455 26 **Wang CJ, Stratmann J, Zhou ZG, Sun XF.** Suppression of microRNA-
456 31 increases sensitivity to 5-FU at an early stage, and affects cell migration and
457 invasion in HCT-116 colon cancer cells. *BMC Cancer* 2010; 10:616

458 27 **Christopoulos P, Pfeifer D, Bartholome K et al.** Definition and
459 characterization of the systemic T-cell dysregulation in untreated indolent B-cell
460 lymphoma and very early CLL. *Blood* 2011; 117:3836-46

461 28 **Lu L, Luo ST, Shi HS et al.** AAV2-mediated gene transfer of VEGF-
462 Trap with potent suppression of primary breast tumor growth and spontaneous
463 pulmonary metastases by long-term expression. *Oncol Rep* 2012; 28:1332-8

464 29 **Ragan C, Zuker M, Ragan MA.** Quantitative prediction of miRNA-
465 mRNA interaction based on equilibrium concentrations. *PLoS Comput Biol* 2011;
466 7:e1001090
467

LEGENDS TO ILLUSTRATIONS

Figure 1. Study outline. Tissue samples were obtained from a total of 150 UTUC patients. Samples were divided into a screening (18 samples) and discovery phase (132 samples). miRNAs differentially expressed between progressing and non-progressing UTUC patients were first identified in the screening phase using the TaqMan® Human MicroRNA Array. **Eight of the miRNAs with false discovery rate (FDR) ≤10% and absolute fold change (FC) ≥ 2 between distinct groups were selected for technical validation in the same cohort using a different platform**. **This was followed by a search to identify genetic UTUC prognostic markers by analyzing five miRNAs that had gave p<0.05 or/and absolute FC ≥ 2 between distinct groups in the results from the technical validation.** None of the samples from the screening set were employed for the genetic markers discovery process.

Figure 2. miRNAs differentially expressed between progressing and non progressing UTUC patients. (A) Heat map displaying the 26 miRNAs differentially expressed (FDR<10) between progressing and non-progressing UTUC patients in the TaqMan® Human MicroRNA Array (n=18). Normalization was carried out with the global mean method. Red pixels correspond to an increased abundance of miRNA in the urine samples, whereas green pixels indicate decreased mRNA levels. Rows represent individual miRNAs and columns represent experimental samples. (B) List of the differentially expressed miRNAs and their fold change values.

Abbreviations: Pool=Pools of primers (Pool A and Pool B) for reverse transcription and preamplification (Applied Biosystems); p value=Student's *t* test *P* values; FC=Fold change values were generated from the median expression of the miRNAs from the TaqMan® Human MicroRNA Array in the groups compared. FDR=False Discovery Rate. Statistical significance FDR<10%.

Figure 3. Kaplan-Meier curves for tumour progression and cancer-specific survival. (A) Kaplan-Meier estimates of probability of being free of tumour progression according to the identified model including miRNA-149 and miRNA-31 expression. Blue line represents low risk patients (RS<0.86; **n=108**), and red line high risk patients (RS≥0.86; **n=39**). **(B)** Kaplan-Meier estimates of probability of cancer-specific survival according to miRNA-149 expression. Blue line represents low risk patients (RS<0.93; **n=96**), and red line high risk patients (RS≥0.93; **n=51**).

TABLES AND THEIR LEGENDS

Table 1. Pathological features of UTUC patients.

		Hospital Clinic of Barcelona	Claude Huriez Hospital of Lille	Pitié Salpétrière Hospital of Paris	Total (%)
N Total		78	56	16	150 (100)
Histological Grade	**I**	6	7	1	14 (9.3)
	II	34	11	3	48 (32)
	III	38	38	12	88 (58.7)
Pathological Stage	**pTa**	9	13	4	26 (17.3)
	pT1	20	17	5	42 (28)
	pT2	17	7	4	28 (18.7)
	pT3	18	17	3	38 (25.3)
	pT4	14	2	0	16 (10.7)

Table 2. Technical validation of TaqMan Array data using LNA primers in the same cohort of patients (n=18).

miRNA	TaqMan Array (GM)		LNA primers (miRNA-218)	
	FC	p value	FC	p value
hsa-miR-141	-3.38	0.01*	-1.37	0.26
hsa-miR-149	-7.89	0.003*	-5.02	0.06
hsa-miR-181a-2*	-2.88	0.03*	-2.09	0.03*
hsa-miR-200a	-2.90	0.01*	1.06	0.77
hsa-miR-31	-7.56	0.01*	-2.16	0.45
hsa-miR-378*	-7.23	0.003*	4.47	0.05
hsa-miR-493	4.42	0.006*	8.52	0.03*
hsa-miR-99a	2.67	0.01*	3.12	0.01*

Abbreviations: p value=Student's *t* test *P* values; GM= global mean normalization method; miRNA-218=data normalization with miRNA-218; FC=Fold change. *Statistically significant ($p<0.05$).

Table 3. Univariate analysis of predictors of tumour progression and cancer-specific survival

	TUMOUR PROGRESSION			CANCER SPECIFIC-SURVIVAL		
	HR	**95% CI**	**p value**	**HR**	**95% CI**	**p value**
miR-149	0.77	0.66 - 0.90	0.0011*	0.80	0.68 - 0.94	0.0071*
miR-181a.2	0.80	0.67 - 0.96	0.0139*	0.81	0.67 - 0.98	0.0337*
miR-31	0.85	0.80 - 0.90	<0.0001*	0.96	0.88 - 1.05	0.3735
miR-493	0.96	0.84 - 1.10	0.5516	0.93	0.81 - 1.06	0.2922
miR-99a	0.97	0.83 - 1.13	0.6767	1.00	0.85 - 1.17	0.9844
Pathological Stage	2.82	2.02 - 3.93	<0.0001*	1.88	1.39 -2.55	<0.0001*
Histological Grade	3.52	1.70 - 7.31	0.0007*	2.29	1.25 - 4.21	0.0075 *

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval. * Statistically significant ($p<0.05$).

Table 4. Altered predicted KEGG pathways in tumour progression and cancer specific survival by miR-31 and miR-149.

KEGG Pathway	p value
Progression	
T cell receptor signaling pathway -hsa04660	0.028
B cell receptor signaling pathway -hsa04662	0.006
GnRH signaling pathway -hsa04912	0.034
ErbB signaling pathway -hsa04012	0.026
Gap junction - hsa04540	0.033
Epithelial cell signaling in Helicobacter pylori -hsa05120	0.008
VEGF signaling pathway -hsa04370	0.000
Adherens junction -hsa04520	0.013
Survival	
Methane metabolism -hsa00680	0.000
Heparan sulfate biosynthesis -hsa00534	0.029
Vitamin B6 metabolism- hsa00750	0.038

Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; hsa value, KEGG reference of each pathway

Figure 1

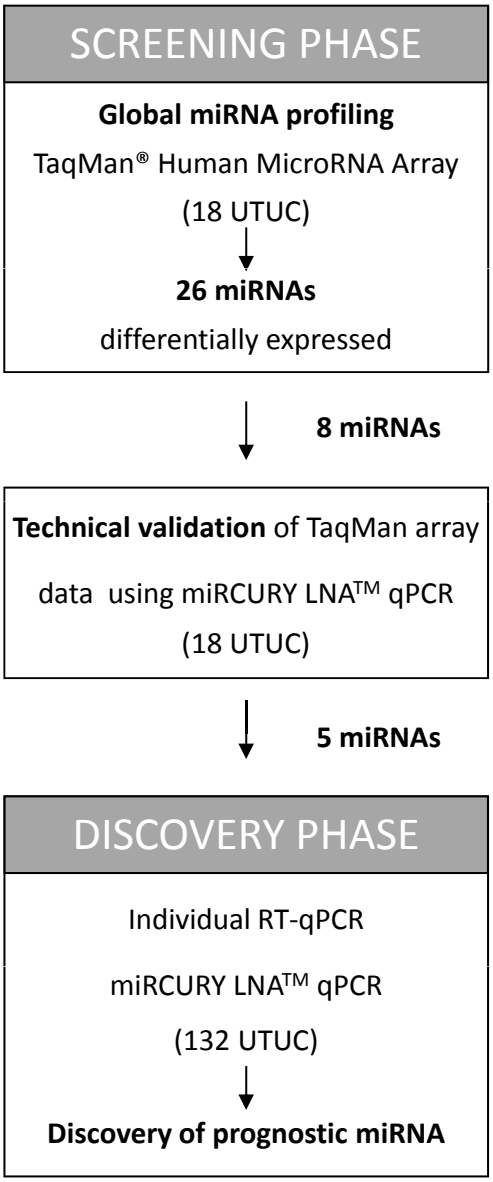
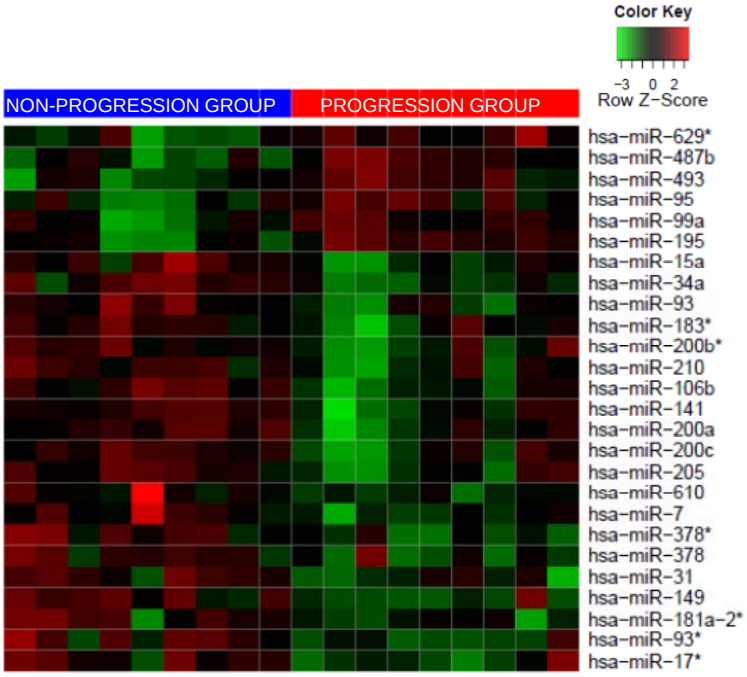

SCREENING PHASE
Global miRNA profiling
TaqMan® Human MicroRNA Array
(18 UTUC)
26 miRNAs
differentially expressed
8 miRNAs
Technical validation of TaqMan array
data using miRCURY LNA™ qPCR
(18 UTUC)
5 miRNAs
DISCOVERY PHASE
Individual RT-qPCR
miRCURY LNA™ qPCR
(132 UTUC)
Discovery of prognostic miRNA

Figure 2

A



B

miRNA	pool	p value	FC	FDR
hsa-miR-31	A	0.011	-7.56	0
hsa-miR-34a	A	0.001	-2.67	0
hsa-miR-141	A	0.011	-3.38	0
hsa-miR-149	A	0.003	-7.89	0
hsa-miR-200a	A	0.011	-2.90	0
hsa-miR-205	A	0.013	-3.51	0
hsa-miR-378*	B	0.004	-7.23	0
hsa-miR-93*	B	0.004	-6.32	0
hsa-miR-7	B	0.014	-2.07	0
hsa-miR-15a	A	0.011	-1.92	5.319
hsa-miR-106b	A	0.002	-1.96	5.319
hsa-miR-200c	A	0.011	-3.45	5.319
hsa-miR-210	A	0.014	-2.91	5.319
hsa-miR-610	B	0.043	-2.10	5.319
hsa-miR-99a	A	0.012	2.67	6.383
hsa-miR-195	A	0.002	1.44	6.383
hsa-miR-487b	A	0.003	4.73	6.383
hsa-miR-493	A	0.006	4.42	6.383
hsa-miR-629*	B	0.005	2.89	6.383
hsa-miR-17*	B	0.022	-2.92	6.383
hsa-miR-95	A	0.011	3.52	8.326
hsa-miR-200b*	B	0.038	-2.31	8.704
hsa-miR-181a-2*	B	0.026	-2.88	8.704
hsa-miR-93	A	0.007	-1.40	9.820
hsa-miR-183*	B	0.044	-1.95	9.820
hsa-miR-378	B	0.043	-3.56	9.820

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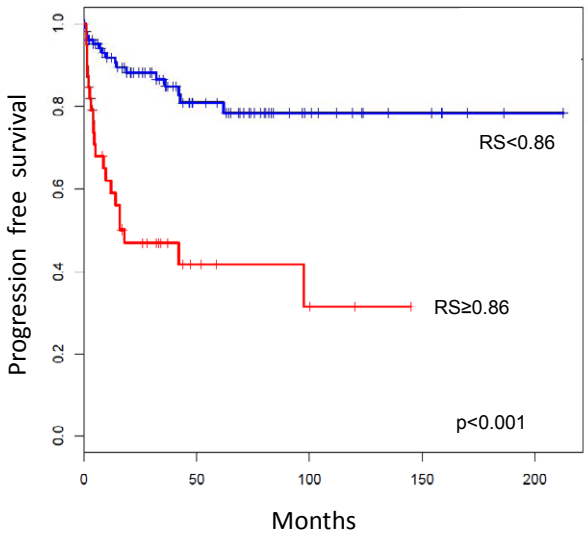
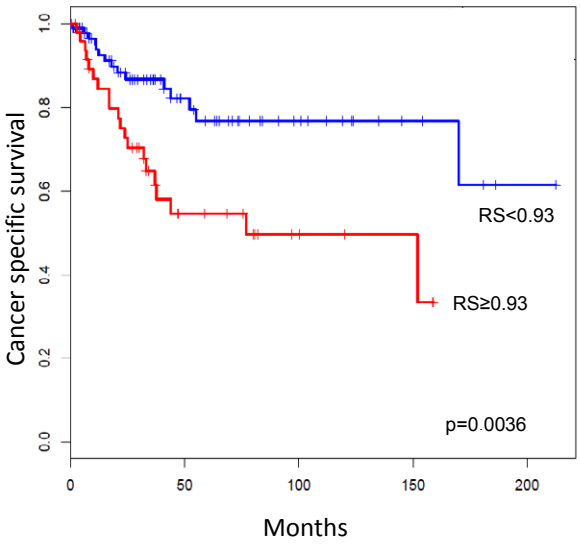
Figure 3

A



B



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