Prognostic value of microRNA expression pattern in upper tract urothelial carcinoma

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

Patients and Methods
We conducted a multicentre, retrospective study of formalin-fixed paraffin-embedded tissue samples from 150 patients with UTUC who had undergone radical nephroureterectomy.

Global miRNA expression patterns were analysed in 18 selected samples from patients with UTUC using TaqMan arrays.

The differential expression of five key miRNAs was validated by quantitative polymerase chain reaction in an independent cohort of 132 samples from patients with UTUC.

Models to predict tumour progression and cancer-specific survival that included miRNA expression patterns were developed by Cox regression analysis.

Results
Twenty-six miRNAs were found to be aberrantly expressed between samples from patients with progressing and non-progressing UTUC 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 derived from these miRNA models were able to discriminate two groups with a highly significantly different probability of tumour progression (hazard ratio [HR] 4.78; P < 0.001) and death (HR 276; P = 0.004).

Conclusions
There is a differential miRNA expression pattern between patients with progressing and non-progressing UTUC.

The identification of new miRNAs associated with a high probability of tumour recurrence and cancer-specific survival in patients with UTUC and their combination in a robust, easy-to-use and reliable algorithm may help tailor treatment and surveillance strategies in these patients.

Keywords
microRNA, prognosis, quantitative 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 outcomes of patients with UTUC [2]. Predictive tools such as nomograms have been proposed after RNU but they are still lacking high accuracy [3]. 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 patients with UTUC 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.

In the present study, we aimed to examine, for the first time, the miRNA expression profiles of patients with progressing and non-progressing UTUC to identify putative miRNAs that may be used as prognostic markers.

**Patients and Methods**

**Patients**

We conducted a retrospective study in a total of 150 patients with UTUC (mean [range] age: 70 [45–101] years, women: n = 34), who underwent RNU at one of three centres (the Hospital Clinic of Barcelona, Barcelona, Spain, the Pitié Salpêtrière Hospital, Paris, France and Claude Huriez Hospital, Lille, France) between 1990 and 2004. The only exclusion criterion was lack of tissue from the archive blocks. The patients’ pathological characteristics are shown in Table 1. Tumours were graded and classified according to the WHO [9] and the TNM classification system of the Union Internationale Contre le Cancer [10]. Tissue samples were obtained with institutional review board approval.

The median (range) follow-up of the studied population was 46 (3–213) months. All patients were followed up postoperatively at 3-month intervals for the first year, at 6-month intervals for the next 2 years, and annually thereafter. Tumours were considered to be progressing when distant metastasis or pathological nodes were developed during the follow-up.

**Tissue Specimens and RNA Isolation**

Once obtained, tissue was fixed in 10% formalin within 24 h and subsequently embedded in paraffin. A slide of each specimen was stained with haematoxylin and 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, Austin, TX, USA) according to the manufacturer’s instructions. Total RNA was quantified by spectrophotometric analysis at 260 nm (NanoDrop Technologies, Wilmington, DE, USA).

**Global Screening Phase**

A flow chart of the entire study is shown in Fig. 1. Global miRNA profiling of 18 randomly selected UTUC cases from the Hospital Clinic of Barcelona, nine progressing (pTa, n = 1; pT1, n = 1; pT2, n = 1; pT3, n = 4; pT4, n = 2; G1, n = 7; G2, n = 1; G3, n = 1; mean progression time 17.2 months) and nine non-progressing (pTa, n = 1; pT1, n = 6; pT2, n = 1; pT3, n = 1; G2, n = 6; G3, n = 3; mean follow-up 94 months), was performed using TaqMan® Human MicroRNA Array A+B Cards Set v2.0 that contain 754 unique assays specific to human miRNAs and four control assays in each card (Applied Biosystems, Foster City, CA, USA) according to manufacturer’s instructions. Briefly, miRNAs were reverse transcribed using the TaqMan miRNA reverse transcription kit (Applied Biosystems) using 500 ng of total RNA following the manufacturer’s instructions (Megaplex RT primers for Human Pool A and B; Applied Biosystems). After reverse transcription, 2.5 μL of cDNA was preamplified with Megaplex PreAmp primers according to the manufacturer’s instructions (Applied Biosystems), except that the final volume of the reaction was

| Pathological features of patients with UTUC. | Hospital Clinic of Barcelona | Claude Huriez Hospital of Lille | Pitié Salpêtrière Hospital of Paris | Total, n (%) |
|---|---|---|---|---|
| No. of patients | 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) |
Study outline. Tissue samples were obtained from a total of 150 patients with UTUC. Samples were then divided into a screening (18 samples) and discovery phase (132 samples). miRNAs differentially expressed between progressing and non-progressing patients with UTUC were first identified in the screening phase using the TaqMan Human MicroRNA Array. Eight of the miRNAs with FDR ≤10% and absolute fold change (FC) of ≥2 between distinct groups were selected for technical validation in the same cohort of patients (Table 2). We also decided to further analyse miR-31, miR-493, miR-99a, miR-378*, miR-181a-2*, miR-149, and miR-149-2 for validation in the 18 previously analysed samples, using a miRCURY Locked Nucleic Acid Universal RT microRNA PCR kit (Exiqon, Vedbaek, Denmark).

We performed reverse transcriptase (RT)-qPCR reactions 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. The 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-149-2 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 Pitie Salpetriere 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). We also decided to further analyse miR-31 and an absolute fold change (FC) of ≥2 between distinct groups in the TaqMan Human MicroRNA Array (n = 21). We then checked whether these differentially expressed miRNAs belonged to the same miRNA cluster, and only one miRNA from each was then selected for further validation (For example clusters hsa-miR-200a, hsa-miR-200b, hsa-miR-200a*, hsa-miR-200b* or hsa-miR-141, hsa-miR-141, hsa-miR-141* containing several differentially expressed miRNAs from our list). Finally, a total of eight differentially expressed miRNAs obtained in TaqMan Human MicroRNA Array were selected for validation in the 18 previously analysed samples, using a miRCURY Locked Nucleic Acid Universal RT microRNA PCR kit (Exiqon, Vedbaek, Denmark).

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

| miRNA     | TaqMan Human MicroRNA Array (GM) | LNA primers (miRNA-218) |
|-----------|----------------------------------|-------------------------|
|           | FC                               | P                       | FC | P       |
| 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† |

P values: Student’s t-test. GM: Global mean normalization method; miRNA-218: data normalization with miRNA-218. †Significant (P < 0.05).
and miR-149 expression in the independent cohort because, although their expression was not significantly different 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 significant differences should be analysed with that in mind, and furthermore, we were searching for biomarker predictors of tumour progression and cancer-specific survival, and it has been previously reported that discrimination rather than significance is the important characteristic of a biomarker [11].

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

Data Analysis

TaqMan Human MicroRNA Array

The RT-qPCR data were processed using SDS 2.4 and ENTERPRISE software packages (Applied Biosystems). 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 with the global mean Cq values were identified and miR-218 was selected by using GeNorm as reference miRNA [13].

Those miRNAs with Cq values >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 were expressed as ΔCq (ΔCq = CqmiR-218–Cqtarget miRNA). miRNAs with Cq values >35 were considered as poorly expressed, and their ΔCq values were imputed to a minimum ΔCq value for that miRNA. FC 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 patients with progressing and non-progressing UTUC were explored using the Student’s t-test. Significance was defined as FDR values <10%. R-software was used for all calculations and to construct a heat map.

miRNA LNA real-time RT-qPCR

No miRNAs with Cq values >35 were found when using LNA primers. Samples with a miR-218 Cq value >30 were considered to have low RNA quality and were excluded from the analysis. ΔCq values were calculated as described above.

Statistical Analysis

Univariable 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 an 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 Σβixi, where i = 1, . . . , k index variables, βi represents the coefficient for each variable estimated from the Cox regression model, and xi is the corresponding value for each variable in a given patient. RS was subjected to a receiver–operator curve analysis to choose the most appropriate threshold for predicting tumour progression and cancer-specific survival; thereafter, Kaplan–Meier curves were generated using the selected threshold and compared according to the log-rank test. As progression and time of death data were 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, the analysis of TaqMan Human MicroRNA Array-derived expression data from nine progressing and nine non-progressing cases resulted in the identification of 26 miRNAs with a FDR of <10%; 20 down-regulated and six up-regulated miRNAs in samples from deceased with respect to live patients. A heat map based on differentially expressed miRNAs shows a distinction between the samples from patients with progressing and non-progressing UTUC (Fig. 2).

Technical Validation of Differentially Expressed miRNAs

To ensure consistency in the experimental procedures followed in the screening phase, we used a different approach, based on LNA 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 FC direction when analysed with LNA PCR primer sets and in three of them (miR-181a-2*, miR-493 and miR-99a) these differences were significant (P < 0.05; Table 2).

miRNAs Associated with Tumour Progression and Cancer-Specific Survival

To identify the miRNAs that correlated with patients’ progression and survival, the expression levels of five key
miRNAs, were analysed using 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 from UTUC. The 5-year tumour progression and cancer-specific survival rates of the series were 71.67 and 70.13%, respectively. The mean times 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 patients’ progression and survival, the miRNAs and the clinical variables in all 132 patients were analysed using a Cox regression model. First, the univariate analysis showed significant predictors of tumour progression and cancer-specific survival (Table 3). Second, the multivariate regression analysis showed that pathological tumour stage and expression of miR-31 and miR-149 were independent prognostic factors of tumour progression (hazard ratio [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; \( P < 0.001 \) and HR 0.82; \( P = 0.018 \), 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 (range) value of this RS was 0.574 (0.043–10.98); thereafter, a receiver–operator curve analysis allowed us to select a threshold of 0.93 to classify patients into a high-risk group of tumour progression (73%). Figure 3A shows the Kaplan–Meier curves generated using the selected threshold. As shown, a 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 (median [range] RS 0.784 [0.188–2.431]). The subsequent receiver–operator curve analysis allowed us to select a threshold 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, \( P = 0.004 \) [Fig. 3B]).

**Discussion**

Approximately 30% of patients with UTUC analysed in the present study died from their tumour after 5-year of follow-up. Pathological stage and histological grade are the established prognostic factors for UTUC but they are insufficient to predict individual tumour behaviour. It would therefore be worth finding more reliable and individualized prognostic markers. To this end, different molecular markers have been previously evaluated in samples from patients with UTUC by using immunohistochemistry [15–21] or in situ hybridization [22], but none of them have been incorporated into the clinical setting. We have also previously analysed the gene expression patterns of several genes in UTUC samples but we were not able to identify the prognostic factors of UTUC based on the genes analysed [15].

miRNAs have been described as novel prognostic molecules involved in several tumours [4–7], but, to our knowledge, miRNA expression profiles of patients with progressing and non-progressing UTUC had not been explored as yet. In the present study, we first investigated global miRNA expression patterns in tissue samples from a reduced cohort of patients with UTUC. We identified a list of 26 miRNAs differentially

| miRNA   | HR   | 95% CI     | \( P \)  |
|---------|------|------------|---------|
| miR-149 | 0.77 | 0.66–0.90  | 0.001†  |
| miR-181a-2* | 0.80 | 0.67–0.96  | 0.014†  |
| miR-31  | 0.85 | 0.80–0.90  | <0.001† |
| miR-493 | 0.96 | 0.84–1.10  | 0.552   |
| miR-99a | 0.97 | 0.83–1.13  | 0.677   |
| Pathological stage | 2.82 | 2.02–3.93 | <0.001† |
| Histological grade | 3.52 | 1.70–7.31 | 0.001†  |

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

**Fig. 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 patients at low risk (RS <0.86; \( n = 108 \)), and red line patients at high risk (RS ≥0.86; \( n = 39 \)). (B) Kaplan–Meier estimates of probability of cancer-specific survival according to miRNA-149 expression. Blue line represents patients at low risk (RS <0.93; \( n = 96 \)), and red line patients at high risk (RS ≥0.93; \( n = 51 \)).
expressed between patients with progressing and non-progressing UTUC, but we were aware that, although global miRNA expression profiling of UTUC samples provides miRNAs implicated in UTUC progression, these were early data that needed 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 the present study were real-time PCR-based, methodological differences between both qPCR platforms such as the priming system for reverse transcription, the use 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, to identify miRNAs that correlate with tumour progression and shorter survival, we tested these five miRNAs in an independent, larger, multicentre cohort of patients with UTUC. As shown, tumour stage and miR-31 and miR-149 expression independently predict tumour progression and, in addition, tumour stage and miR-149 expression independently predict cancer-specific survival. As a result, the RS derived from miRNAs in our multivariate model was able to discriminate two groups with a highly significantly different probability of tumour progression (HR 4.78, \( P < 0.001 \)) and cancer-specific survival (HR 2.76, \( P = 0.004 \)); 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. The analysis of these two miRNAs in UTUC tissue samples refines the currently used clinico-pathologically 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-cell RCC, squamous cell carcinoma of the tongue, prostate cancer, glioblastoma and astrocytoma. In addition, it has been reported to serve as a diagnostic and prognostic marker for bladder and colorectal cancer [24,25]. Meanwhile, 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 significantly 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, especially haematological and breast neoplasms [27,28].

Regarding the application of miRNAs in the clinical setting, it is interesting 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, while short mature miRNAs are more stable against nuclease degradation because of their smaller size and, in fact, the isolation of high-quality mRNA from formalin-fixed paraffin-embedded 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 to be ~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 the present study is based on our use of a non-targeted, exploratory approach to select the candidate miRNAs. Furthermore, we used archival formalin-fixed paraffin-embedded samples to obtain miRNA expression patterns allowing an easy translation of the results obtained to clinical practice. Finally, a multicentre cohort with prospective data collection and long-term follow-up is analysed in the present study, which eliminates the limitations of a single-population study. We are aware, however, that the study has some limitations. 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 excluded some miRNAs highly predictive of tumour progression and cancer-specific survival. Second, although we tried to include a substantial number of patients from three different centres, the total number of patients analysed can

| KEGG pathway                                      | \( P \)     |
|--------------------------------------------------|------------|
| T cell receptor signalling pathway: hsa04660      | 0.028      |
| B cell receptor signalling pathway: hsa04662      | 0.006      |
| GnRH signalling pathway: hsa04912                 | 0.034      |
| ErB2 signalling pathway: hsa04012                 | 0.026      |
| Gap junction – hsa04540                           | 0.033      |
| Epithelial cell signalling in Helicobacter pylori: hsa05120 | 0.008      |
| VEGF signalling pathway: hsa04370                 | 0.000      |
| Adherens junction: hsa04520                       | 0.013      |

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

Table 4 Altered predicted Kyoto Encyclopedia of Genes and Genomes pathways in tumour progression and cancer-specific survival by miR-31 and miR-149.
still be considered low. Third, 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 warrant further prospective evaluation in carefully and specifically designed studies, the present study may contribute to the identification of a reliable prognostic system for patients with UTUC.

In conclusion, our results demonstrate that there is a differential miRNA expression pattern between patients with non-progressing and progressing UTUC. We also show that an algorithm that combines miR-31 and miR-149 expression is able to 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, the identification of new miRNAs associated with a high probability of tumour recurrence and cancer-specific survival in patients with UTUC and its inclusion in a robust, easy-to-use and reliable algorithm may help tailor treatment and surveillance strategies in these patients.

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Conflict of Interest
None declared.

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Abbreviations: miRNA, microRNA; UTUC, upper tract urothelial carcinoma; RNU, radical nephroureterectomy; qPCR, quantitative PCR; Cq, cycle quantification; FDR, false discovery rate; FC, fold change; LNA, locked nucleic acid; RS, risk score; HR, hazard ratio.