RESEARCH ARTICLE

Rapid detection of urinary long non-coding RNA urothelial carcinoma associated one using a PCR-free nanoparticle-based assay

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Abstract

We developed a specific hybridization assay for direct detection of long non-coding RNA urothelial carcinoma associated-1 (lncRNA-UCA1). Total RNA was extracted from urine pellet samples (bladder carcinoma patients and controls). Then, we compared the developed nanoassay with quantitative real time polymerase chain reaction (qRT-PCR) results in detection of urine UCA1 in bladder cancer and control samples. The sensitivity and the specificity of UCA1 nanoassay were 92.1% and 93.3%, respectively. The concordance of the two methods was 98%. Interestingly, all bilharzial benign cases showed negative lncRNA-UCA1 using both methods. UCA1-nanoassay is a valid test for direct detection of urine UCA1 for bladder cancer detection.

Keywords

Bilharziasis, bladder cancer, gold nanoparticles, long non-coding RNA-UCA1, urine biomarkers, urine cytology

Introduction

To date, bladder cancer (BC) is one of the most common malignant tumors among males in Egypt and some of the Middle East and African countries (Khaled, 2013). Therefore, there is an urgent need to develop reliable molecular markers and therapeutic targets against metastasis and relapse of bladder carcinoma (Kamat et al., 2013).

Among the long non-coding RNAs (lncRNAs, >200 bp), lncRNA-UCA1 particularly aroused our attention due to its significant upregulation in BC, which is closely associated with the proliferation, metastasis and drug resistance of bladder cancer cells (Fan et al., 2014). UCA1 is believed to exert its functions as a ncRNA in the regulation of embryonic development and BC invasion and progression (Srivastava et al., 2014).

Nanoparticle-based assays have been shown to have enhanced specificity and sensitivity as compared to conventional methods (Baptista, 2012). Moreover, in contrast to the majority of micro- and macroparticles, nanoparticles (NPs) have highly significant properties, such as surface plasmon resonance (SPR), which have enabled the development of new diagnostic strategies (Li et al., 2012).

Herein, we present Au-NP-based approach for the molecular detection of UCA1 to improve the diagnosis of BC, which is inexpensive, very easy to perform and uses total human RNA as a target without reverse transcription or amplification steps. The developed UCA1 Au-NPs assay was compared to quantitative real time polymerase chain reaction (qRT-PCR). Finally, the correlation between urine lncRNA-UCA1 expression and bilharziasis was addressed.

Patients and methods

Research ethics statement

The study was approved by the Research Ethics Committee of Faculty of Medicine, Ain Shams University, Egypt. A written informed consent was obtained from all participants for the use of their urine and blood samples in this study.

Patients selection

The study included 220 Egyptian subjects: 184 patients who were admitted to Urology Department, Ain Shams University, Egypt, in addition to 36 healthy normal volunteers as a control group who were recruited from the hospital laboratory staff, between January 2013 and June 2014. Patients who received any previous chemotherapy, with past history of bladder cancer or any other malignancy within the past five years were excluded from the study (Table 1). Biopsy of any suspicious lesion was performed for histopathological examination. Tumor samples were staged according to the American Joint Committee on Cancer-Union and Internationale Contre le Cancer tumor-node metastasis classification (Edge et al., 2010; NCCN Clinical Practice Guidelines in Oncology: Bladder Cancer, 2013) and histologically graded by an expert uropathologist (Eble et al., 2004).
The benign group included 45 patients with benign urological diseases who underwent cystoscopy for bilharzial dysplastic lesions, benign prostatic hyperplasia and urethral stricture.

Schistosomal antibodies were detected in sera using a Cellogent Schistosomiasis H kit supplied by Dade Behringwerke AG (Marburg, Germany) (Gui et al., 1991). Around 40 ml of voided urine samples were obtained before cystoscopy or transurethral resection of the tumor. After centrifugation at 1000 g, a portion of the pellet was used for cytology examination and the remaining portion was treated with RNA later (Qiagen Inc., Chatsworth, CA) and stored at 80°C for further processing.

Extraction of total RNA, including IncRNA

Urine pellet was processed using the RNeasy Mini Kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer’s instructions. The concentration of RNA was measured spectrophotometrically.

cDNA synthesis and quantitative real-time PCR analysis

cDNA was synthesized using a QuantiTect® Reverse Transcription kit (Qiagen Inc., Chatsworth, CA) in Hybaid thermal cycler (Thermo Electron, Waltham, MA) according to the manufacturer's instructions. The expression of IncRNAs-UCA1 was measured by TaqMan probe qPCR using the specific primer/probe combination provided with each TaqMan assay (Hs_UCA1_QF_1 QuantiFast Probe Assay, Qiagen Inc.); and TaqMan Universal PCR Master Mix on StepOnePlus™ System (Applied Biosystems Inc., Foster City, CA) according to the manufacturer’s instructions (Accession NR_015379.3). Hs_GAPDH_QF_2 QuantiFast Probe Assay (Qiagen Inc.) was used as an internal control (Accession NM_004360), and each sample was analyzed in triplicate (Supplementary Figure 1A and B). Relative gene expression (fold change) was calculated using the 2^-ΔΔCt method (Livak & Schmittgen, 2001).

Antisense oligonucleotide primer sequence and Au-NP synthesis

The antisense primer sequence (5'-GCGGCAGGTCTTAAAGAGATGAG-3') (Wang et al., 2008b) was quoted from previous Pubmed literatures. The 13 nm gold nanoparticles were prepared by the citrate reduction method described by Lee and Meisel (Lee & Meisel, 1982).

Au-NP hybridization and color detection of urine IncRNA-UCA1

Assay solution containing the Au-NP and target RNA was prepared by mixing various concentrations of the total RNA (final concentration 50–200 ng/μl), from each sample, with the hybridization buffer (Eissa et al., 2014; Shawky et al., 2010). One antisense primer targeting IncRNA-UCA1 (oligo-target) was mixed with NaCl solution. Different salt and primer concentrations were tested to determine their optimal concentrations for the assay. The hybridization buffer contained NaCl and anti-sense primer with final concentrations...
of 0.56 M and 12 μM, respectively. The Au-NP assay was performed in a total volume of 40 μl by mixing 5 μl hybridization buffer, 5 μl sample RNA and 30 μl Au-NP. The color change was observed within 1 min. Blank measurements were made in exactly the same conditions, but without target or total RNA which was replaced with an equivalent volume of 10 mM phosphate buffer (pH 8.0). Each assay was repeated at least three times for each RNA extract. The photographs were taken 1 min after the addition of the Au-NPs.

**Determination of sensitivity and specificity**

A serial dilution of IncRNA-UCA1 (ranging from 10 to 100) was analyzed to determine the lowest concentration at which a positive signal could be detected (limit of detection; LOD). The specificity of the assay was evaluated by testing cross-reactivity when RNA which was extracted from urine samples, that exhibited positive nanoassay, was treated with Dyna beads® Oligo (dT)25 (Life Technology, Milan, Italy). The latter can only target and capture mRNA molecules but cannot capture IncRNAs in any sample.

**Statistical analysis**

All statistical analyses were performed using the SPSS18.0 software (SPSS Inc., Chicago, IL). The results were presented as means ± standard deviation. Differences between means were analyzed using the unpaired Student’s t-test (two-tailed) or One Way ANOVA test as appropriate. Univariate analyses were performed using a Chi-square test for association of categorical variables. The cutoff value for the qPCR analysis was calculated using ROC curve. The probability value \( p < 0.05 \) was considered statistically significant.

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### Results

**Urine IncRNA-UCA1 expression by qRT-PCR**

The clinical characteristics of the participants and the pathology of cancers are listed in Table 1. We performed qPCR using Taqman probe-based method to assess the differential expression of IncRNA-UCA1 in urine; thus, validating its clinical utility for BC diagnosis. As summarized in Table 2, the mean relative quantity (RQ) of IncRNA-UCA1 in the urine samples was: 0.91, 0.99 and 1.3 in healthy donors, benign and malignant groups, respectively. The detected IncRNA-UCA1 level was significantly lower in healthy donors and benign groups compared to bladder cancer samples.

ROC curve analysis, based on RQ values, was performed to assess the sensitivity and specificity of the IncRNA-UCA1 RT-qPCR assay in distinguishing bladder cancer patients from non-malignant group. The obtained 95% CI was equal to (0.945–0.987) and the AUC was 0.966. When the cutoff value was set to the optimal point, 1.09, the obtained specificity was 93.3%, sensitivity was 89.2% and positive predictive value was 97.6% (Tables 3 and 4, Figure 1). We found significant correlation between the IncRNA-UCA1 level by qPCR and bilharziasis, pathological type, stage and grade of the tumor \( (p < 0.05) \) (Supplementary Table 1).

**IncRNA-UCA1 Au-NPs assay: performance characteristics**

In negative samples the test color remained red; while in positive samples the color changed from red to blue (Figure 2). One hundred and twenty-eight malignant samples out of 139 were positive using the IncRNA-UCA1 Au-NP assay, with a sensitivity of 92.1%. On the other hand, all

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**Table 2. Bladder cancer-related urine UCA1 RNA differential expression by qRT-PCR among the different investigated groups.**

|            | N  | Mean | Std. deviation | Std. error | 95% Confidence interval for mean | Lower bound | Upper bound | Minimum | Maximum |
|------------|----|------|----------------|------------|---------------------------------|-------------|-------------|---------|---------|
| Malignant  | 139| 1.3  | 0.45           | 0.038      |                                 | 1.23        | 1.38        | 1.02    | 4.2     |
| Benign     | 45 | 0.99 | 0.15           | 0.023      |                                 | 0.95        | 1.04        | 0.089   | 1.1     |
| Normal     | 36 | 0.91 | 0.31           | 0.051      |                                 | 0.811       | 1.017       | 0.06    | 1.09    |

Significant difference was detected between investigated groups at \( p < 0.001 \) using One Way ANOVA test based on relative quantity (RQ) value \( (F = 3.2 \text{ at } p < 0.0001) \).

**Table 3. The frequency of positive cytology and UCA1 detected by investigated methods in urine samples of the studied groups \( (n = 220) \).**

|            | Malignant | Benign | Normal |
|------------|-----------|--------|--------|
| Cytology\(^a\) | Positive  | 69 (49.6%) | 1 (2.2%) | 0 (0%) |
|             | Negative  | 70 (50.4%) | 44 (97.8%) | 36 (100%) |
| UCA1 RNA qRT-PCR\(^b\) | Positive  | 124 (89.2%) | 3 (6.7%) | 0 (0%) |
|             | Negative  | 15 (10.8%) | 42 (93.3%) | 36 (100%) |
| UCA1 RNA Au-NPs assay\(^c\) | Positive  | 128 (92.1%) | 3 (6.7%) | 0 (0%) |
|             | Negative  | 11 (7.9%) | 42 (93.3%) | 36 (100%) |

Significant difference was detected between the investigated groups at \( p < 0.001 \) using Chi-square test. Au-NPs: gold nanoparticles, qRT-PCR: quantitative real time polymerase chain reaction, UCA1: urothelial cancer associated 1.

\(^a\chi^2 = 55.3 \text{ at } p < 0.0001.\)

\(^b\chi^2 = 153.69 \text{ at } p < 0.0001.\)

\(^c\chi^2 = 166.3 \text{ at } p < 0.0001.\)
36 normal samples were negative, as well as 42 out of 45 benign samples. Three benign samples were false-positive using the nanoassay. The calculated specificity of the developed assay was 93.3% (Tables 3 and 5). We also evaluated the specificity of the developed assay by comparing the results of Au-NP assay using RNA samples extracted by two different methods namely RNeasy Mini Kit for total RNA extraction; and Dynabeads® Oligo(dT)25 for mRNA extraction. Au-NP assay showed negative results with Dynabeads® Oligo(dT)25 and positive results with RNA extracted by RNeasy Mini Kit. The lower detection limit of the lncRNA-UCA1 Au-NPs assay was 1.3 nmol/l. The assay turnaround time was about 30 min. There was no significant correlation between urine lncRNA-UCA1 expression results detected by Au-NP assay and any of the clinicopathological factors of bladder cancer patients.

Table 4. Correlation between UCA1 RNA status which was detected by the investigated methods and the different clinicopathological factors of bladder cancer patients.

|                | qPCR                  | Cases ≥ cutoff | Positive nanoassay<sup>b</sup> (n = 128) | Positive cytology<sup>b</sup> (n = 69) |
|----------------|-----------------------|----------------|------------------------------------------|----------------------------------------|
| **Groups (n = 139)** | **Mean ± SD<sup>a</sup>** | **Positive<sup>c</sup> (n = 124)** | **Positive<sup>c</sup> (n = 128)** | **Positive<sup>c</sup> (n = 69)** |
| **Age**        |                       |                |                                          |                                        |
| ≥61 years (n = 69) | 1.3 ± 0.5             | 61 (49.2%)     | 63 (49.2%)                               | 42 (60.9%)                             |
| <61 years (n = 70) | 1.2 ± 0.3             | 63 (50.8%)     | 65 (50.8%)                               | 27 (39.1%)                             |
|                | 0.57                  | 0.8            | 0.7                                      | 0.009**                                |
| **Sex**        |                       |                |                                          |                                        |
| Male (n = 107)  | 1.26 ± 0.3            | 97 (78.2%)     | 100 (78.1%)                              | 55 (79.7%)                             |
| Female (n = 32) | 1.4 ± 0.7             | 27 (21.8%)     | 28 (21.9%)                               | 14 (20.3%)                             |
|                | 0.051                 | 0.3            | 0.27                                     | 0.45                                   |
| **Smoking**    |                       |                |                                          |                                        |
| Positive (n = 70) | 1.3 ± 0.4             | 64 (51.6%)     | 65 (50.8%)                               | 33 (47.8%)                             |
| Negative (n = 69)| 1.3 ± 0.5             | 60 (48.4%)     | 63 (49.2%)                               | 36 (52.2%)                             |
|                | 0.57                  | 0.39           | 0.7                                      | 0.55                                   |
| **Bilharziasis** |                       |                |                                          |                                        |
| Positive (n = 41) | 1.4 ± 0.5             | 41 (33.1%)     | 41 (32%)                                | 19 (27.5%)                             |
| Negative (n = 98)| 1.3 ± 0.4             | 83 (66.9%)     | 87 (68%)                                | 50 (72.5%)                             |
|                | 0.36                  | 0.008**        | 0.02*                                    | 0.6                                    |
| **Pathological type** |                   |                |                                          |                                        |
| TCC (n = 112) | 1.3 ± 0.5             | 103 (83.1%)    | 105 (82%)                               | 45 (65.2%)                             |
| SCC (n = 27)   | 1.2 ± 0.2             | 21 (16.9%)     | 23 (18%)                                | 24 (34.8%)                             |
|                | 0.28                  | 0.03*          | 0.139                                    | 0.001**                                |
| **Clinical stage** |                       |                |                                          |                                        |
| Stage (I) (n = 69) | 1.2 ± 0.12            | 62 (50%)       | 64 (50%)                                | 27 (39.1%)                             |
| Stage (II) (n = 42) | 1.2 ± 0.17            | 36 (29%)       | 38 (29.7%)                              | 24 (34.8%)                             |
| Stage (III) (n = 28) | 1.6 ± 0.9             | 26 (21%)       | 26 (20.3%)                              | 18 (26.1%)                             |
|                | 0.001**               |                | 0.83                                    | 0.041*                                 |
| **Histological grade** |                   |                |                                          |                                        |
| 1 (n = 24)     | 1.3 ± 0.17            | 22 (17.7%)     | 23 (18%)                                | 9 (13%)                                |
| 2 (n = 79)     | 1.25 ± 0.15           | 72 (58.1%)     | 73 (57%)                                | 39 (56.5%)                             |
| 3 (n = 36)     | 1.47 ± 0.8            | 30 (24.2%)     | 32 (25%)                                | 21 (30.4%)                             |
|                | 0.04*                 | 0.4            | 0.613                                   | 0.29                                   |
| **Urinary cytology** |                       |                |                                          |                                        |
| Positive (n = 69) | 1.2 ± 0.15            | 60 (48.4%)     | 62 (48.4%)                              | –                                      |
| Negative (n = 70) | 1.4 ± 0.6             | 64 (51.6%)     | 66 (51.6%)                              | –                                      |
|                | 0.014*                | 0.39           | 0.33                                    | –                                      |
| UCA1 ≥ cutoff by qRT-PCR | 1.3 ± 0.47          | –              | 124 (96.9%)                             | 60 (87%)                               |
| Positive (n = 124) | 1.06 ± 0.02           | –              | 4 (3.1%)                                | 9 (13%)                                |
| Negative (n = 15) | 0.02*                 | –              | 0.001**                                 | 0.39                                   |
| UCA1 by nanoassay |                       |                |                                          |                                        |
| Positive (n = 128) | 1.3 ± 0.46            | 124 (96.9%)    | –                                        | 62 (48.4%)                             |
| Negative (n = 11) | 1.06 ± 0.02           | 4 (3.1%)       | –                                        | 66 (51.6%)                             |
|                | 0.055                 | 0.001**        | –                                        | 0.33                                   |

Au-NPs: gold nanoparticles, qRT-PCR: quantitative real time polymerase chain reaction, UCA1: urothelial cancer associated 1, TCC: transitional cell carcinoma; SCC: squamous cell carcinoma.

<sup>a</sup>Significant difference was detected between investigated groups at p < 0.05 using independent t-test or One Way Anova test<sup>a</sup> and Chi-square test<sup>b</sup>.

<sup>b</sup>Highly significant difference at p < 0.01.

Figure 1. ROC curve analysis for lncRNA-UCA1 to calculate the best cutoff point that discriminates between the malignant and non-malignant groups. Best cutoff point of lncRNA-UCA1 is 1.09 (sensitivity = 89.2% and specificity = 93.3%). Area under the curve (AUC) [SE] = 0.966 [0.011], 95% confidence limits range = 0.945–0.987, p < 0.0001).
malignant samples, except bilharziasis (Supplementary Table 1).

Concordance of results of urine IncRNA-UCA1 detected by Au-NP assay and cytology in all investigated groups

The developed IncRNA-UCA1 assay was significantly superior to urine cytology. It was positive in 66 malignant cases (51.6%) with negative urine cytology signifying its role in early detection of bladder cancer. The combination of results of the developed nanoassay and urine cytology improved the sensitivity of urine cytology from 49.6% to 97.1%, even in low grade and superficial bladder cancer (Table 4).

Relation of IncRNA-UCA1 expression by qRT-PCR or Au-NP assay to bilharziasis

This study showed a significant difference in IncRNA-UCA1 expression between bilharzial benign and malignant cases by both assays. Interestingly, all bilharzial benign cases showed negative IncRNA-UCA1 as shown in Supplementary Table (2).

Discussion

A critical factor in the management of bladder cancer is the early detection using highly sensitive diagnostic techniques, especially with the limited sensitivity of classical cytology in low grade tumors (Smith et al., 2014).

IncRNA-UCA1 has been reported to play a pivotal role in bladder cancer progression and could serve as a promising biomarker for diagnosis, prognosis and therapeutic targeting for bladder cancer (Wang et al., 2006; Wang et al., 2008a). Ectopic expression of IncRNA-UCA1 in bladder cancer cell line, significantly promoted tumorigenicity and invasive potential of cells. Other studies found that IncRNA-UCA1 is associated with tumor-linked genes, such as WNT6 and BRG1 (Peifer & Polakis, 2000; Wang et al., 2014; Xue et al., 2014).

We have adopted an assay for direct detection of unamplified IncRNA-UCA1 in pellet of urine samples, using gold nanoparticles to aid in bladder cancer diagnosis (Eissa et al., 2014; Shawky et al., 2010). The optimized assay resulted in a reproducible and accurate detection of unamplified urine IncRNA-UCA1 even in samples with minimal urine volume, and/or low RNA concentration.

Moreover, we detected IncRNA-UCA1 using qRT-PCR with 89.2% sensitivity and 93.2% specificity. The introduction of

### Table 5. Performance characteristics of the assays used for urinary UCA1 RNA detection and cytology in bladder cancer group, superficial (stage 0 + I) and in Grade 1 bladder cancer.

| Parameter                          | Sensitivity (%) | Specificity (%) | NPV (%) | PPV (%) | Accuracy (%) |
|------------------------------------|----------------|----------------|---------|---------|--------------|
| Bladder cancer group (n = 139)     |                |                |         |         |              |
| Cytology                           | 49.6           | 98.75          | 53      | 98.5    | 70.4         |
| qRT-PCR UCA1                       | 89.2           | 96.25          | 83.7    | 97.6    | 92.7         |
| UCA1 by nanoassay                  | 92.1           | 96.25          | 87.5    | 97.7    | 93.2         |
| qRT-PCR UCA1 + cytology            | 95.7           | 96.25          | 92.8    | 97.8    | 95.5         |
| UCA1 by nanoassay + cytology       | 97.1           | 96.25          | 95      | 97.8    | 96.4         |
| Superficial bladder cancer (stage I)(n = 69) | | | | | |
| Cytology                           | 39.1           | 98.75          | 65.3    | 96.4    | 69.7         |
| qRT-PCR UCA1                       | 89.9           | 96.25          | 91.7%   | 95.4    | 93.3         |
| UCA1 by nanoassay                  | 92.8           | 96.25          | 93.9    | 95.5    | 94.6         |
| qRT-PCR UCA1 + cytology            | 94.2           | 96.25          | 95      | 95.6    | 95.3         |
| UCA1 by nanoassay + cytology       | 97.1           | 96.25          | 97.5    | 95.7    | 96.7         |
| Grade 1 bladder cancer (n = 24)    |                |                |         |         |              |
| Cytology                           | 37.5           | 98.75          | 84      | 90      | 84.6         |
| qRT-PCR UCA1                       | 91.7           | 96.25          | 97.5    | 88      | 95.2         |
| UCA1 by nanoassay                  | 95.8           | 96.25          | 98.7    | 88.5    | 96.2         |
| qRT-PCR UCA1 + cytology            | 91.7           | 96.25          | 97.5    | 88      | 95.2         |
| UCA1 by nanoassay + cytology       | 95.8           | 96.25          | 98.7    | 88.5    | 96.2         |
| Bilharzial bladder cancer(n = 41)  |                |                |         |         |              |
| Cytology                           | 46.3           | 98.75          | 78.2    | 95      | 81           |
| qRT-PCR UCA1                       | 100            | 96.25          | 100     | 93      | 97.5         |
| UCA1 by nanoassay                  | 100            | 96.25          | 100     | 93      | 97.5         |
| qRT-PCR UCA1 + cytology            | 100            | 96.25          | 100     | 93      | 97.5         |
| UCA1 by nanoassay + cytology       | 100            | 96.25          | 100     | 93      | 97.5         |

Reference method is cystoscopy. Au-NPs: gold nanoparticles, qRT-PCR: quantitative real time polymerase chain reaction, UCA1: urothelial cancer associated 1.
Au-NPs assay, increased the sensitivity of lncRNA-UCA1 detection to 92.2% with the same specificity. In addition, Au-NPs assay was more advantageous with regard to cost and simplicity.

Although, urine cytology remains the corner stone test for BC diagnosis and follow-up, the developed lncRNA-UCA1 Au-NPs assay is significantly superior to urine cytology. The combination of the developed assay with urine cytology improved the sensitivity of urine cytology even in low grade and superficial bladder cancer.

As a matter of fact, lncRNAs were shown to play important roles in mediating the host-Schistosome interaction (Oliveira et al., 2011). Similarly, we found a significant difference between bilharzial benign and malignant cases regarding urinary lncRNA-UCA1 expression, in the event that bilharzial infestation might play a role in the aberrant expression of urine lncRNA-UCA1 underlying carcinogenesis.

Conclusion

To our knowledge, this is the first report on the detection of human long non-coding RNA directly from total RNA without reverse transcription or amplification. The assay has a comparable sensitivity to those demonstrated by qPCR, and the total cost of one sample assay was calculated to be around $8.0. Equally important, lncRNA-UCA1 Au-NP assay can detect bilharzial and non-bilharzial bladder cancer at high sensitivity and specificity. Further studies are needed to validate the current method in a multicentric study. In addition, a follow-up of bilharzial cases is required to understand the association of lncRNA-UCA1 expression and BC development.

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Declaration of interest

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The authors declare that they have no competing interests.

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Supplementary material available online

Supplementary Table 1 and 2, Figures 1a and 1b