Editorial Commentary

Circular RNAs as diagnostic tool for renal transplant patients with acute rejection

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The kidney is a multifunctional organ that filters waste products from the blood in our body in the form of urine. In addition, kidneys also function to regulate blood pressure, balance electrolytes, and secrete erythropoietin to support erythrocyte (red blood cells) production. Severe loss of kidney function or kidney failure results in the accumulation of fluid and metabolic waste products—if left untreated, can lead to seizures, coma, or even death. Currently, the gold standard treatment for end-stage kidney disease patients is kidney transplantation (1). Unfortunately, as of 2016, the numbers of kidneys available for kidney transplantation are less than 17% of what are needed (2,3). Hence significant effort had gone into preventing transplant rejection or transplant failure due to reoccurrence of the primary disease or the development of graft interstitial fibrosis and glomerulosclerosis (4). In effort to avoid such complication and support graft survival in recipients, patients must take immunosuppressive medications. When to start these medications depends on the severity of acute kidney rejection, which is classically measured by kidney allograft biopsy (5,6). However, renal biopsy is invasive and carries potential associated risks to patient health. Non-invasive methods are preferred, which is a focus of intensive research in recent years (7-14).

Alternative splicing ("splicing") is a post-transcriptional process in which exons of nascent precursor messenger RNA (pre-mRNA) transcripts are included or excluded to form the mature mRNA (15). In humans, more than 90% of multi-exonic genes undergo splicing (16,17). Due to its important contribution in diversifying protein isoforms from one gene, dysregulation in splicing (called "aberrant splicing") results in various human diseases, including renal diseases (18-21). Recent studies show that some spliced out exons and introns are not degraded in the nucleus, but an upstream 3' splicing site (ss) can join with a downstream 5' ss in a reversed order (called "backsplicing") to give rise to circular RNAs (circRNAs) (22-24). Since their discovery in early 1990s, studies have shown that circRNAs are quite stable and predominantly localize to the cytoplasmic compartment of cells (25,26). One interesting feature of circRNAs is that they are more stable than that of non-circular RNAs (e.g., mRNA); this is due to the fact that circRNAs, which lack free 5' and 3' ends, are not susceptible to RNA exonuclease-mediated degradation. Because of their stability, circRNAs accumulate in the blood (27-29), saliva (30), and urine (31), which have made them attractive candidates for biomarker discovery. Due to the less invasive (i.e., collection of blood) and non-invasive (i.e., saliva and urine) procedures necessary for their collection from patients, as compared to surgically procured/needle biopsy samples, circRNAs stand as an easily accessible diagnostic biomarker to potentially distinguish/identify various types of human diseases.

In a recent study by Kölling *et al*. (32), authors investigated

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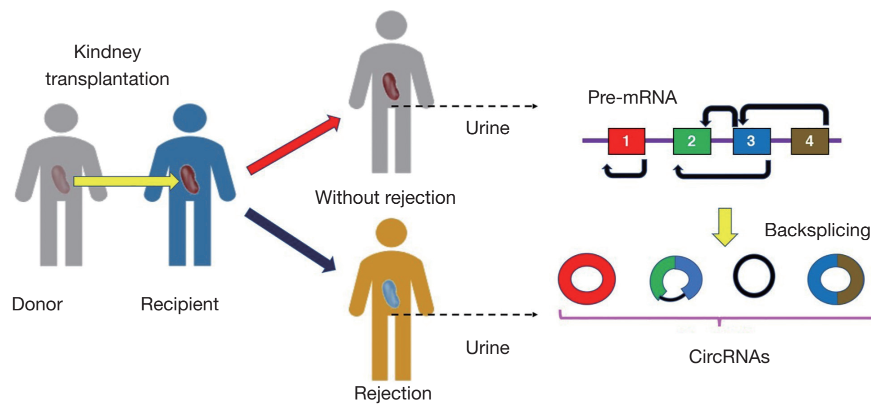

Figure 1 Detection of circRNAs from urine. Because circRNAs are more stable than linear mRNAs, Kölling *et al.* (32) proposed the usage of circRNAs in urine to distinguish kidney transplant patients with acute T cell-mediated allograft rejection from those without rejection.

the potential of using circRNAs in urine to distinguish kidney transplant patients with acute T cell-mediated allograft rejection from those without rejection (*Figure 1*). By utilizing human circRNA microarray (targeting 13,617 circRNAs), the authors detected 5,199 circRNAs in urine. Of these, 363 circRNAs were identified to be differentially expressed. Among these differentially expressed circRNAs, the authors chose two circRNAs, *hsa_circ_0071475* and *hsa_circ_0001334*, for further analysis.

According to circBase (33), the database for circRNAs, *hsa_circ_0071475* is located on chr 4:183,245,098-183,268,082 (GRCh37/hg19) and spans exons 1 and 2 of teneurin transmembrane protein 3 (*TENM3*) gene, which encodes for a transmembrane protein to regulate eye (34) and neuronal development (35). This circRNA was originally identified in Salzman *et al*. (36) and Rybak-Wolf *et al*. (37) to be expressed in cell lines (e.g., Hela, human ES cells) and various regions of brain (e.g., cerebellum, frontal cortex), respectively. Similarly, *hsa_circ_0001334* is located on chr 3:127,337,917-127,341,124 (GRCh37/hg19) and spans exons 13 to 16 of minichromosome maintenance complex component 2 (*MCM2*) gene, which is involved in the initiation of genome replication (38). This circRNA was previously reported to be detected in $CD19^+$ leukocytes (39). Since it is known that the expression pattern and function of circRNA may correlate with its parental gene (40), it is interesting to note that previous studies reported *MCM2* can be used as prognostic proliferative marker in renal cell carcinoma (41) and Wilms tumor (42), suggesting that the selected circRNA, *hsa_circ_0001334*, can be originated from kidneys. Indeed, further validation experiments by Kölling *et al*. (32) show that the concentrations of *hsa_circ_0001334* (but not *hsa_circ_0071475*) were significantly increased in patients with acute T cell-mediated rejection compared with stable transplant controls without signs of rejection. To further confirm the specificity of this differential expression, the authors also provide an evidence that the concentrations of this circRNA did not differ in kidney transplant patients with urinary tract infection. Based on these expression analyses, the authors propose *hsa_circ_0001334* as a biomarker of acute renal allograft rejection, which can be detected in urine of patients to offer a non-invasive method.

As noted by the authors, it is a single-center cohort study. Thus, more rigorous, multi-center studies are needed to further confirm the validity of *hsa_circ_0001334* as a biomarker. Given that circRNAs can be detected by RT-PCR assay, the primer pair can be designed at the backsplicing site to specifically target the circRNA but not its parental gene. Thus, the multi-center studies can be easily conducted using urine. Furthermore, the molecular mechanism of urinary circRNA release is not provided in this study, which is harder to investigate as the origin of circRNAs is extremely difficult to detect unless the parental gene is cell-type specifically expressed (more so than tissue specificity). However, as noted above, previous studies indicate the upregulation of *MCM2*, the parental gene of *hsa_circ_0001334*, in renal tumors (41,42). Thus, it is plausible that aberrant splicing of *MCM2* gene caused by deterioration in transplanted kidney may produce *hsa_circ_0001334*. However, a closer investigation about splicing variants of MCM2 gene is needed as there are nine MCM2 transcripts annotated in the Ensembl database (Ensembl Gene ID: ENSG00000073111), including five protein-coding, two nonsense mediated decay, one retained

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intron, and two lncRNAs. One lncRNA, *MCM2-203* (Ensembl Transcript ID: ENST00000468659.1), spans between exons 12 and 16 of the longest isoform of MCM2 gene, *MCM2-201* (Transcript ID: ENST00000265056.12), which corresponds to the majority of region covered by *hsa_circ_0001334*. As with any other lncRNA and circRNA research, rapid amplification of cDNA ends (RACE) and Northern blotting experiments must be performed to understand the whole transcriptomic length of *hsa_circ_0001334* to delineate its distinction from another lncRNA encoded by the parental gene, MCM2.

In the last part of the Results section, the authors provide the *in silico* screening data of possible binding of miRNAs to *hsa_circ_0001334*. However, no direct, biological evidence (e.g., binding assay between circRNA and miRNAs) has not been provided, which raises a question about *hsa_circ_0001334* functioning as miRNA sponges. Indeed, a comprehensive bioinformatics analysis (43) and our biological validation experiments (44) indicate that circRNAs acting as miRNA sponges are rare, while binding to RNA-binding proteins (RBPs) is more frequent (45-47) as some RBPs [e.g., Muscleblind (48), Quaking (49)] are involved in the biogenesis of circRNAs. Although it is outside of the scope of the current study, further detailed studies, including the characterization of *hsa_circ_0001334* for its exact transcript length and biogenesis as well as gain/loss-of-function experiments, are needed to understand the biology of circRNA in general.

In conclusion, the study by Kölling *et al*. (32) provides a nice addition to a repertoire of circRNAs detected in urine, which could serve as biomarkers for various diseases and their progressions, including acute renal allograft rejection investigated in this study.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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