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# Free microRNA levels in plasma distinguish T-cell mediated rejection from stable graft function after kidney transplantation

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ABSTRACT

The potential diagnostic value of circulating free miRNAs in plasma compared to miRNA expression in blood cells for rejection processes after kidney transplantation is largely unknown, but offers the potential for better and timely diagnosis of acute rejection. Free microRNA expression of specific blood cell markers was measured in 160 plasma samples from kidney transplant patients under standard immunosuppressive therapy (steroids ± mycophenolic acid ± calcineurin inhibitor) with stable graft function, urinary tract infection, interstitial fibrosis and tubular atrophy, antibody-mediated rejection (ABMR), Borderline (Banff3), tubulo-interstitial (Banff4-I) and vascular rejection (Banff4-II/III) applying RT-PCR. The expression levels of specific microRNAs miR-15B, miR-103A and miR-106A discriminated patients with stable graft function significantly (p-values 0.001996, 0.0054 and 0.0019 resp.) from patients with T-cell mediated rejection (TCMR) and from patients with urinary tract infection (p-values 0.0001, <0.0001 and 0.0001, resp.). A combined measurement of several microRNAs after multivariate logistic regression improved the diagnostic value supported by subsequent cross-validation. In conclusion, the measurement of circulating microRNAs in plasma from patients with renal transplants distinguishes TCMR and urinary tract infection from stable graft function. In contrast to miRNA expression measurement in blood cells it does not allow a discrimination from ABMR or interstitial fibrosis and tubular atrophy.


## 1. Introduction

All types of graft rejection, as well as infections and interstitial fibrosis/tubular atrophy (IFTA) are still common problems after transplantation and may cause impaired graft function and survival. A highly sensitive and specific diagnosis with fast and non-invasive methods is inevitable to provide the adequate treatment in time thereby preventing long-term graft loss. Serum creatinine is an acceptable measure for monitoring kidney function, but subclinical disease processes may lead to delayed diagnosis and the pathohistological examination of biopsy material still provides the most reliable diagnostic results. Unfortunately this procedure cannot be performed frequently. So the search for biomarkers after kidney transplantation continues in order to discover means that allow the prediction, diagnosis and monitoring of particular conditions of the graft [1]. Expression levels of RNAs are altered in many diseases and also in the course of medical events after renal transplantation. The class of small non-coding RNAs, including microRNAs, qualify as robust biomarkers after renal transplantation, because they are present and highly stable in body fluids [2].

MicroRNAs fine tune gene expression and therefore have an impact on various cellular functions including proliferation, differentiation and apoptosis. They have been widely analyzed regarding their potential as biomarkers in medical conditions like cancer [3–5], multiple sclerosis [6] and also kidney disease [7]. Focusing on kidney pathologies, the expression of microRNAs has for example been linked to functional consequences in the pathogenesis of polycystic kidney disease [8], chronic kidney disease [9], lupus nephritis [10] and diabetic nephropathy [11–12]. MiRNA regulation has especially been studied in complications after renal transplantation, including acute T-cell mediated rejection [13–17], antibody-mediated (ABM) rejection [18] and chronic allograft dysfunction with IFTA [19–20]. Those microRNA studies have been

*Abbreviations:* ABMR, antibody-mediated rejection; AUC, area under the ROC curve; BL, borderline; CNI, calcineurin-inhibitor; CV, cross-validation; DSA, donor-specific antibodies; IFTA, interstitial fibrosis/tubular atrophy; MPA, mycophenolic acid; ROC, receiver operating characteristics; TCMR, T-cell mediated rejection; UTI, urinary tract infection.

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performed with a wide variety of source materials, such as tissue, plasma, serum, urine and peripheral blood cells.

The finding of our recent study, that the measurement of 5 specific microRNAs in blood cells identifies T-cell mediated vascular rejection (Banff4-II/III) [21], constitutes the basis for the presented work. We focused on the microRNAs with the highest diagnostic value for TCMR in blood cells and measured the plasma expression of miR-15B, miR-16, miR-103A and miR-106A in a large kidney transplanted patient cohort with stable graft function, urinary tract infection (UTI), Banff3-Borderline (BL), Banff4-I cellular rejection, Banff4-II/III cellular rejection, Banff2 ABM rejection, combination of cellular/ABM rejection and Banff5 IFTA. We could show that the expression of miR-15B, miR-103A and miR-106A in plasma discriminates indeed between patients with stable graft function and TCMR, but the diagnostic power is much lower and less specific than the measurement of the 5 microRNAs in whole blood cells.

## 2. Objective

The aim of this study was to assess the diagnostic value of plasma expression of miRNAs that have previously been identified as diagnostic markers for TCMR in blood cells focusing on microRNAs miR-15B, miR-16, miR-103A and miR-106A.

## 3. Materials and methods

### 3.1. Patients and sample collection

Adult renal transplant recipients were recruited from the Department of Nephrology, Campus Mitte, Universitätsmedizin Charité, Germany and provided informed consent. The study was approved by the local ethical committee. 109 plasma samples were collected from 109 patients at the time of indicated biopsy, 40 samples from 40 control patients with stable graft function (no protocol biopsies) and 11 samples from 11 control patients with UTI. Standard immunosuppression consisted of steroids ± calcineurin-inhibitor (CNI) ± mycophenolic acid (MPA). Histology was classified according to the Banff'09/'13 criteria and carried out by two experienced nephropathologists in blinded fashion. 17 patients were diagnosed with Banff3-BL, 15 patients with Banff4-I, 24 with Banff4-II/III, 15 with Banff2-ABM, 6 patients with a combination of cellular/ABM and 32 patients with Banff5-IFTA. Patient demographics are summarized in Table 1, a detailed listing can be found in Supplementary Table I. Patients were tested for DSA by Luminex Single Antigen Beads [22].

### 3.2. Quantification of miRNAs

RNA was isolated from 200 μL plasma with the miRCURY RNA Isolation Kit-Biofluids (Exiqon, Woburn, MA, USA) according to the manufacturer's instruction, eluting with 50 μL and adding 5 nM Syncel-miR-39-3p (Qiagen, Hilden, Germany). RNA concentration and quality of each sample was measured with the NanoDrop ND-Lite (peqlab, Erlangen, Germany). Subsequently, 10 ng RNA was reverse transcribed with the TaqMan® MicroRNA Reverse Transcription Kit (Life Technologies, Darmstadt, Germany) and TaqMan® MicroRNA Assays for candidate miRNAs miR-15B, miR-16, miR-103A, miR-106A, miR-22 and cel-miR-39-3p (Life Technologies, Darmstadt, Germany). TaqMan RT-PCR was performed in duplicate with the assays mentioned above and TaqMan®Universal MasterMixII (Applied Biosystems, Darmstadt, Germany). Cel-miR-39-3p was used as reference gene for normalization given by the formula ($2^{-}\Delta^{C}_{t}$) [23].

### 3.3. Statistical analysis

For analyzing expression data from single miRNAs and to correct for multiple testing, a nonparametric one-way ANOVA (Kruskal-Wallis test) was performed to test whether samples from the 8 different patient groups originate from the same distribution. If significant (p-value < 0.05), a nonparametric Mann-Whitney *U* test was performed between two patient groups (28 comparisons for each single microRNA). The false discovery rate was adjusted using the Benjamini-Hochberg procedure [24].

The diagnostic value of miR-15B, miR-16, miR-22, miR-103A and miR-106A expression levels was evaluated by univariate logistic regressions for each candidate/control miRNA. Receiver operating characteristics (ROC) analysis was applied to determine the Area under the ROC curve (AUC) as well as the sensitivity and specificity at optimal miRNA expression cut-off, defined as the maximal Youden's index [25].

The diagnostic value of a combination of the four candidate miRNAs was evaluated by multivariate logistic regression. For single and combined miRNA expression a 3-fold cross-validation (CV) was performed with 500 repetitions, by keeping the prevalence of the sample sets at the same levels. For variable selection in multivariate logistic analysis the AUC values from the cross validation of the univariate models were used. In particular, miRNA candidates were ranked by the respective AUC values and sequentially removed from the multivariate model. The set of model candidates was extended by a) removal of one or a permutation of two miRNA-candidates and b) using the classical backward elimination process, with a cut-off value of p-value ≥0.25 or p-value ≥0.05 in the Wald-statistics. The optimal variable combination was

**Table 1**
Patient demographics, ns…not specified.

| Group | Recipient age mean; SD | Recipient gender | Serum Cr [mg/dL] mean; SD | Previous kidney Tx | d post Tx mean; SD | Donor gender | Donor age mean; SD | Donor living (l)/non-living (nl) | Donor related r/non-related (nr) |
|---|---|---|---|---|---|---|---|---|---|
| Control | 50,84 | f (14) | 2,01 | 0 (39) | 38,93 | f (22) | 49,98 | nl (20) | r (11) |
| | 17,01 | m (26) | 1,36 | 1 (1) | 56,80 | m (18) | 14,37 | l (20) | nr (9) |
| UTI | 56,17 | f (11) | 2,12 | 0 (11) | 2363,64 | f (3) | 55,2 | nl (7) | r (2) |
| | 15,01 | m (0) | 0,67 | | 2166,20 | m (7) | 8,48 | l (3) | nr (1) |
| | | | | | | ns (1) | | ns (1) | |
| Banff3-BL | 47,11 | f (6) | 2,47 | 0 (15) | 893,00 | f (11) | 48,53 | nl (9) | r (6) |
| | 17,53 | m (11) | 0,88 | 1 (2) | 1394,84 | m (6) | 15,74 | l (8) | nr (2) |
| Banff4-I | 51,84 | f (4) | 3,55 | 0 (14) | 879,67 | f (11) | 55,13 | nl (12) | r (3) |
| | 13,91 | m (11) | 2,36 | 1 (1) | 1260,16 | m (4) | 14,01 | l (3) | nr (0) |
| Banff4-II/III | 51,38 | f (5) | 5,99 | 0 (19) | 386,46 | f (9) | 51,38 | nl (20) | r (1) |
| | 17,28 | m (19) | 4,74 | 1 (3) | 847,98 | m (15) | 18,14 | l (4) | nr (3) |
| | | | | 2 (2) | | | | | |
| Banff2-ABM | 43,82 | f (3) | 2,37 | 0 (13) | 2959,87 | f (8) | 45,33 | nl (8) | r (5) |
| | 9,84 | m (12) | 1,13 | 1 (2) | 2824,50 | m (7) | 15,69 | l (7) | nr (2) |
| Combi | 41,41 | f (2) | 3,60 | 0 (6) | 1222,17 | f (2) | 52,5 | nl (4) | r (2) |
| | 21,72 | m (4) | 3,01 | | 1106,91 | m (4) | 12,49 | l (2) | nr (0) |
| Banff5-IFTA | 56,01 | f (14) | 3,72 | 0 (27) | 2435,81 | f (14) | 56,55 | nl (25) | r (4) |
| | 15,63 | m (18) | 2,51 | 1 (4) | 2453,55 | m (16) | 18,27 | l (5) | nr (1) |
| | | | | 2 (1) | | ns (2) | | ns (2) | |