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ORIGINAL ARTICLE

A noninvasive diagnostic approach to retrospective donor HLA typing in kidney transplant patients using urine

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SUMMARY

Antibody-mediated rejection (AMR) is a major obstacle to long-term kidney transplantation. AMR is mostly caused by donor specific HLA antibodies, which can arise before or any time after transplantation. Incomplete donor HLA typing and unavailability of donor DNA regularly preclude the assessment of donor-specificity of circulating anti-HLA antibodies. In our centre, this problem arises in approximately 20% of all post-transplant HLA-antibody assessments. We demonstrate that this diagnostic challenge can be resolved by establishing donor renal tubular cell cultures from recipient´s urine as a source of high-quality donor DNA. DNA was then verified for genetic origin and purity by fluorescence in situ hybridization and short tandem repeat analysis. Two representative cases highlight the diagnostic value of this approach which is corroborated by analysis of ten additional patients. The latter were randomly sampled from routine clinical care patients with available donor DNA as controls. In all 12 cases, we were able to perform full HLA typing of the respective donors confirmed by cross-comparison to results from the stored 10 donor DNAs. We propose that this noninvasive diagnostic approach for HLA typing in kidney transplant patients is valuable to determine donor specificity of HLA antibodies, which is important in clinical assessment of suspected AMR.

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Key words
antibody-mediated rejection, HLA typing, HLA-antibody post-transplantation, rejection

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Introduction

End-stage renal disease (ESRD) is a diagnosis of profound negative prognosis for the affected individual and high socio-economic impact for the society [1]. For the patient with ESRD as well as the healthcare system, kidney transplantation (KTx) is more beneficial, as compared to dialysis [2]. Therefore, once established long-term maintenance of KTx is crucial. By identification of histological features and development of solid-phase assays for routine testing, antibody-mediated rejection (AMR) has been recognized the leading cause of KTx failure, over the last decade [3,4].

Donor-specific antibodies (DSA) against HLA epitopes can either be performed prior to KTx or acquired years or decades after transplantation, termed *de novo* DSA (dnDSA). The latter appear to be associated with a worse prognosis [5,6]. To date, there is no approved therapy against AMR, although some novel strategies such as IL-6 pathway inhibition, C1 esterase inhibition and enzymatic cleavage of IgG are being tested promisingly [7,8]. In the absence of approved therapies prevention of AMR by stringent HLA matching at time of KTx, sufficient baseline immunosuppression and screening of HLA antibodies may be advantageous [9]. Although, solid phase anti-HLA antibody analysis as a yearly screen after KTx was initially discussed controversially, it has since been widely adopted by many centrs [8,10,11].

Ideally, at the time of transplantation complete donor HLA typing information should be documented for HLA-A, -B, -C, -DRB1/3/4/5; -DQA1/DQB1 and -DPA1/DPB1, since any of these loci can cause deleterious allogenic immunoreactivity [7,12]. In practice, however, this complete data set is not consistently available, e.g. if KTx lies many years back or patients move abroad. In the Eurotransplant region, full typing of the donors for the respective loci have been projected, but still not implemented [13]. As a result, solid-phase immunoassays regularly detect anti-HLA antibodies, for which donor-specificity can not be assigned. Supplemental HLA typing from donor material would therefore be required. However, the aforementioned circumstances often coincide with a lack of donor DNA for typing. Unfortunately, the subset of patients potentially affected by this diagnostic blind spot is hard to quantify as those patients are certainly underreported in the current literature.

In this study, we describe a noninvasive approach to consistently obtain donor material suitable for DNA extraction at any time after transplantation from spontaneous samples of the recipients' urine. The technique for culturing primary tubular cells from the urine was published previously by others [14]. We have recently applied this technique to clarify the genetic background of a kidney donor more than ten years after her death [15]. Very recently another group has used this technique to introduce an interesting method of co-culturing donor tubular cells with recipient´s T cells to monitor allogenic responses [16]. Our study introduces this procedure as a reliable method to retrospectively perform donor HLA typing wherever and whenever necessary. We believe that this method can be a valuable diagnostic application in particular for those KTx patients without stored donor DNA.

Materials and methods

Unless otherwise stated, chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Patient and ethical approval

All patients gave written informed consent to all clinical and scientific procedures. The studies were approved by ethics committee of the Friedrich-Alexander University Erlangen-Nürnberg (approval number: 251_18 B).

Patient and antibody report calculations

All cumulative post-kidney transplantation follow-up antibody reports between 1 July 2018 and 26 August 2020

for the three transplantation centres Erlangen, Regensburg and Würzburg were extracted from the report database of the Laboratory for Immunogenetics Erlangen, including repeat measurements of individual patients. A cumulative count of reports containing detectable anti-HLA antibodies against untyped donor HLA antigens ('ambiguous reports') and reports with either no detectable antibodies or antibodies against known donor antigens ('unambiguous reports') was performed. To establish the number of affected patients, only the status (ambiguous/unambiguous) of the last report on any given patient in the data set was counted ($n = 1415$ patients), thereby avoiding over-representation of ambiguous results by patients for whom ambiguities were resolved within the observed period. Negative antibody tests were also counted as unambiguous.

Histological studies

The Patient A received two kidney biopsies, where the smaller one was extracted for DNA. The larger cylinder was used for routine histopathological assessment.

Cell culture

Human urinary Primary Tubular Cells (huPTC) were cultured from a 50 to 100 ml specimen of spot urine, exactly following a published protocol [14]. In brief, adherent tubular cells were selected by serum free medium in a 12-well culture plate, being the only urinary cell type surviving and proliferating under these conditions. As described, rapid processing of the urinary samples resulted in improved rates of cell viability [14]. When cells approached confluency the huPTC were trypsinized and seeded into a 6-well plate, and then 10 cm^2 culture dish, successively. huPTC were extracted for DNA after the second passage from a confluent 10 cm^2 culture dish, after approximately 2–4 weeks of culture.

Genomic DNA was isolated from blood lymphocytes, tissue culture cells and the kidney biopsy according to standard procedures.

Single antigen bead assay for HLA antibodies

Antibody testing was performed according to the standards of the European Federation for Immunogenetics (EFI). Firstly, sera were tested on generic level for HLA antibodies using LABScreen® Mixed Assay (LSmixed; One Lambda Inc., West Hills, CA, USA) and analysed on a LABScan 200® flow analyzer (One Lambda), applying the manufacturer's recommended threshold ratio of >2.2 for positive results. Secondly, in sera tested positive on generic level, the anti-HLA antibody specificity was determined using a single antigen assay for HLA class I (i.e. HLA-A/B/Cw) and/or HLA class II antigens (i.e. HLA-DR/DQ/DP; LABScreen® Single Antigen Assay, LS1A04 and LS2A01; One Lamba). Tests were carried out according to the manufacturer's instructions. A positive result for antibody specificities in Single Antigen bead array was defined as a baseline normalized MFI >500. Donor-specificity of HLA antibodies were determined via comparison of the HLA-antibody specificities with the donor HLA typing. For every individual DSA, the reported strength was based on the MFI value of the respective antibody.

HLA typing

Donor HLA typing was performed according to the standards of the EFI. HLA typing was tested using RSSO Typing technique on Luminex platform for HLA-A-, HLA-B-, HLA-C-, HLA-DRB1,3,4,5-, HLA DQA1-HLA-DQB1, HLA-DPA1- and HLA-DPB1 antigens (One Lambda Inc.) and analysed on LABScan 3D® flow analyser (One Lambda). The tests were carried out according to the manufacturer's instructions.

Fluorescence in situ hybridization

Cells were spread on Superfrost slides and fixed in a 3:1 methanol/acetic acid solution. Slides were washed in phosphate-buffered saline (PBS) solution for 5 min at room temperature, treated with 0.1% pepsin/0.1N HCl for 5 min at 37 °C, washed with PBS for 5 min and then fixed with 1% paraformaldehyde in PBS for 10 min at room temperature. Subsequently, the slides were washed in PBS and dehydrated in 70%, 90% and 100% ethanol solution for 5 min each at room temperature. Slides were air dried at room temperature for 20 min. Then, fluorescence in situ hybridization (FISH) probe mix XA X/Y/18 (Metasystems, Altlussheim, Germany) was hybridized with the samples overnight at 37 °C. After hybridization, the slides were washed in 0.4× saline sodium citrate buffer (SSC) for 3 min at 75 °C and in 4xSSC/0.1% Tween20 for 5 min at room temperature. Subsequently, DNA was stained with DAPI and the fluorescent signals (centromeric region of chromosomes X in green and Y in red) were captured with a fluorescence microscope (Axioplan2; Zeiss, Jena, Germany). For each sample, 250 interphases of the cultured cells were analysed, and fluorescent images were recorded with a magnification of 630×.

Immunofluorescence staining

Immunofluorescence staining for N-cadherin was performed on hUPTC. Cells were seeded on glass slides and fixed with 4% paraformaldehyd [4% PFA in phosphate-buffered saline (PBS)] for 5 min at room temperature (RT) and permeabilized by 0.5% Triton X-100 in PBS for 10 min. Blocking was performed for 1 h at RT with 1% bovine serum albumine (BSA) in PBS. Subsequently, mouse anti-N-cadherin antibody (#sc-7939, 1:200 in PBS; Santa Cruz Biotechnology, Dallas, TX, USA) was applied overnight at 4 °C and secondary Alexa Fluor 488-conjugated goat anti-mouse antibody (A28175; Thermo Fisher Scientific, Waltham, MA, USA, 1:1000 in PBS) for 1 h at RT. Nuclei were with 4′,6-Diamidin-2-phenylindol (DAPI, 1:1000).

Short tandem repeat analysis

Short tandem repeat (STR) detection of donor and recipient DNA was performed by utilizing the AmpFlSTR™ Identifiler™ Plus PCR Amplification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Analysis was performed with the GeneScan Analysis software (Applied Biosystems, Foster City, CA, USA). Peak intensities are displayed as relative fluorescence units (RFU).

Results

Frequency of assignment difficulties of HLA antibodies due to incomplete donor HLA typing

In order to quantify the diagnostic problem of incomplete information of the donor HLA typing, we analysed our complete data sets of post-transplantation HLA-antibody tests for the last 24 months. Figure 1 shows the antibody test results for 2906 sera (from 1415 patients), 1278 of which carried anti-HLA antibodies detectable by solid-phase assays. Importantly, 605 tests out of 2906 were reported with unknown donor-specificity of detected anti-HLA antibodies (20.8%). Counting individual patients, 21.1% (299/1415) remained with an ambiguous test result during the observed period. The extent of the problem may differ nationally and internationally. However, characteristic cases will always occur in centres, examples of which are highlighted next.

Case histories of representative patients

Case A: The female Patient A escaped from Iraq, arriving in Germany in July 2015. She was not able to carry any medical records on her escape. Her medical history includes a sudden unexpected disease in midst of

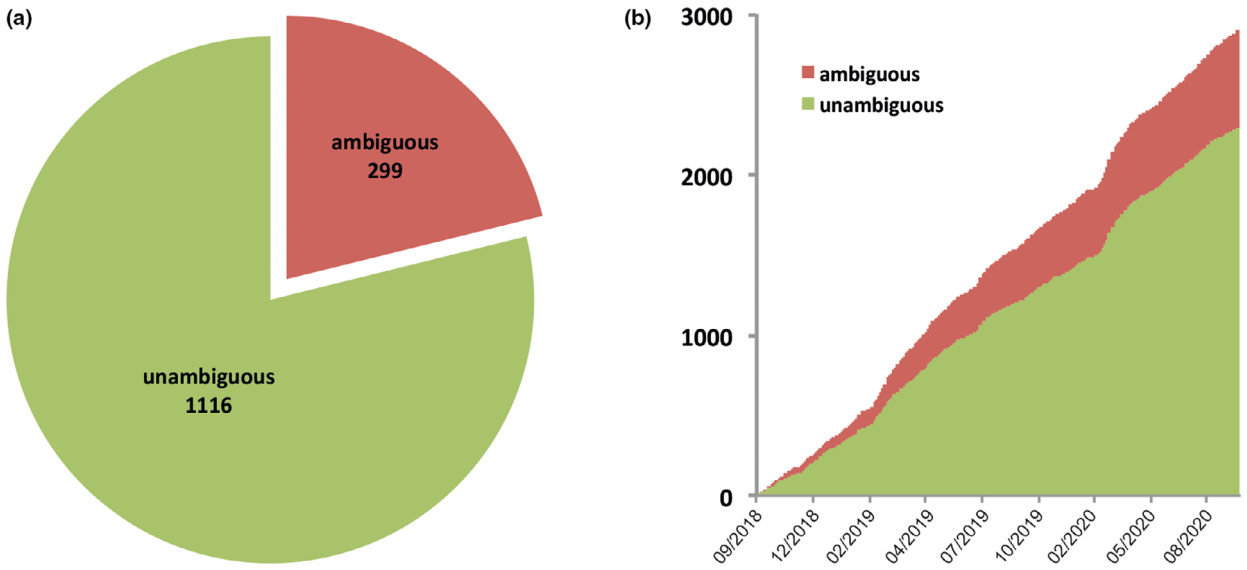

Figure 1 Post-transplant patient and report statistics from the three transplant centres Erlangen, Regensburg and Würzburg between July 2018 and August 2020. (a) Number of unique patients with post-transplant antibody tests with ambiguous and unambiguous specificity of HLA-antibodies due to incomplete donor typing information. (b) Cumulative number of post-transplantation antibody reports with ambiguous and unambiguous assignment of donor specificities during the indicated time period.

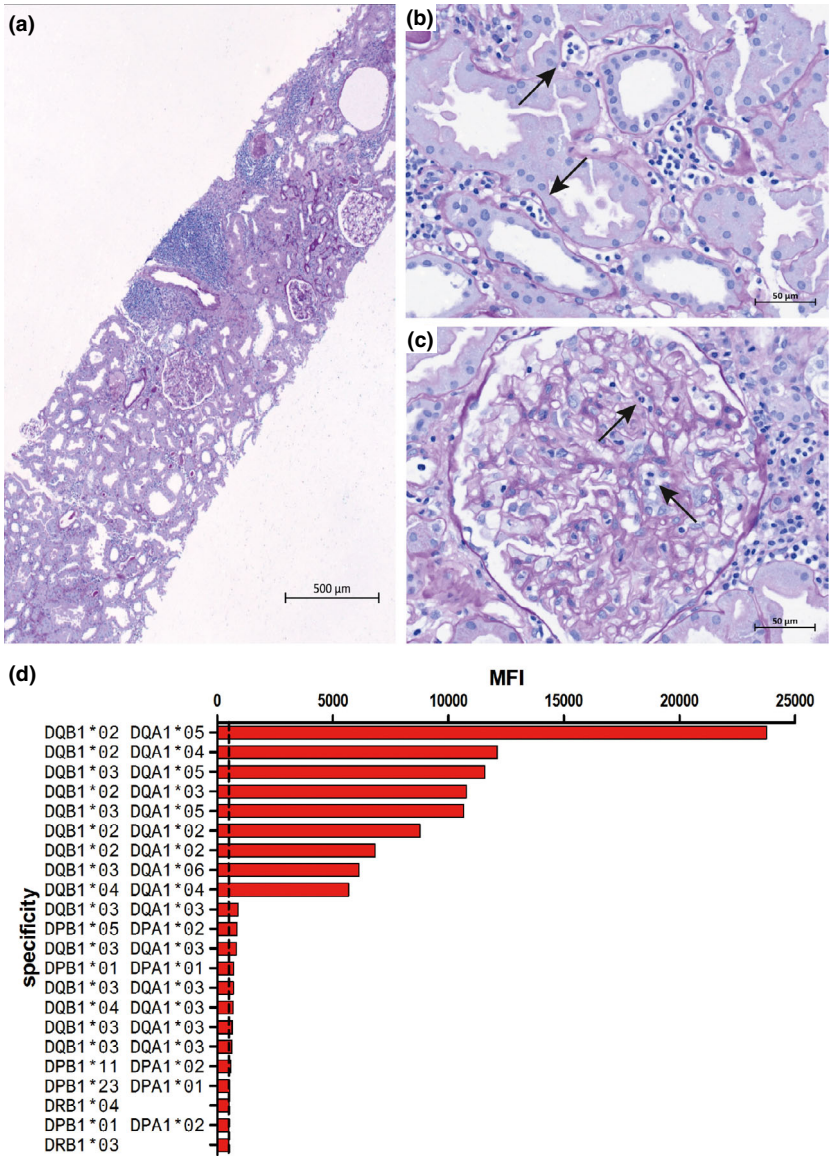

Figure 2 Clinical findings of antibody mediated rejection (AMR). Patient A received a KTx biopsy in 2019 (a–c, stained with Periodic acid-Schiff), where a second biopsy cylinder was retrieved for DNA extraction and further analyses (see Fig. 3). The biopsy showed moderate interstitial fibrosis and tubular atrophy at an extent of approximately 10–15%. Scale bar indicates the magnification (a). Typical features of AMR can be seen, such as peritubular capillaritis (arrows in b) and glomerulitis (arrows in c). Staining for C4d was negative (data not shown). Repeatedly, Patient A was tested for HLA antibodies by single antigen bead technique, which showed numerous specificities in class II for HLA-DQ and HLA-DP (d, HLA results in May 2017). The mean baseline fluorescence intensity (MFI) of positive reactions above the threshold of 500 (dashed line) are shown. *Y*-axis labels are the alpha and beta chain specificities covered by individual beads. In the absence of any knowledge of the donor, the putative relevance of the HLA antibodies could not be determined.

military conflicts in Iraq in 2013, at the age of 24 years. Advanced renal failure was detected and haemodialysis was initiated in June 2013. A kidney biopsy was not performed. The family history was empty for renal disease. Hence, the underlying renal disease was never defined. She received a living, nonrelated kidney donation in December 2013. The immunosuppression consisted of cyclosporine (CYA), myfenolate mofetil (MMF) and steroids. It is not known whether induction therapy was performed. The allograft led to immediate and persistent freedom of dialysis. Her serum creatinine at arrival in Germany was 1.2 mg/dl with no proteinuria.

In 2016, our patient wished to become pregnant and the immunosuppression was reduced to CYA and low-dose steroids. Azathioprine previously resulted in

Table 1. Split-level HLA-types and anti-HLA antibodies (peak MFI in brackets) for cases A and B.

	Case A			Case B	
	Patient	Spouse	Donor	Patient	Donor
A*	01, 02	03, 24	03, 68	03, 24	11, 24
B*	39, 41	13, 49	35, 52	18, 27	27, 49
C*	12, 17	07, 07	04, 12		02, 07
DRB1*	07, 15	03 [DR17], 11	03 [DR17], 13	15, 15	11, 16
DQB1*	03 [DQ9], DQ6	02, 03 [DQ7]	02, 06		03 [DQ7], 05
DQA1*	01, 02	05, 05	01, 05		01, 05
DPB1*	04, 17	04, 04	04, 09		04, 04
DPA1*	01, 02	01, 01	01, 02		01, 01
DRB3/4/5*	4*01 [DR53], 5*01 [DR51]	3*02 [DR52]	3*02 [DR52]	[DR51]	3*02 [DR52], 5*02 [DR51]
Patient antibodies	DR4(521), DR18(501), DQ2(23768), DQA1*03(10783), DQA1*04(12119), DQA1*05(23768), DQA1*06(6140), DP1 (710), DP5(860), DP11(597), DP23(536)			B45(1125), B46(2688), DR1(550), DR4(676), DR8(862), DR9(780), DR11(535), DR12(1007), DR14 (1083), DR15(580), DR16(634), DR51(510), DR52(864), DP1(1026), DP3(664), DP5(679), DP10(1068), DP11(850), DP13(856), DP15(1212), DP19(2064), DP20(1273), DP28 (1010), DQA1*03(1134), DQA1*06 (619)	

pancreatitis and had to be withdrawn. In 2017, she lost two pregnancies due to spontaneous abortions at 12 weeks and two weeks, respectively. In succession, her creatinine rose to a maximum of 1.8 mg/dl and transplant biopsies were performed in 2017 and 2019, showing typical signs of acute AMR (Fig. 2a–c). HLA antibodies were analysed for the first time in 2017 showing a number of high intensity class II antibodies for HLA-DQ (Fig. 2d). Since we had no information on the kidney donor in Iraq, definition of specificity of the antibodies could not be performed. The immunosuppression was converted to Tacrolimus (TAC) and MMF was re-instituted, which stabilized the creatinine to approximately 1.4 mg/dl until today.

Case B: Patient B is a female from Romania, who seeks medical advice in our clinic 1–2 times per year. She suffers from systemic Lupus Erythematodes, which led to kidney failure and need for haemodialysis in 2005, at the age of 26 years. She received a kidney transplant by live donation of a cousin in Bukarest in 2008. Immunosuppression was maintained with TAC, MPA and steroids throughout. The transplant function was excellent up until recently, with creatinine serum concentration below 1 mg/dl and no proteinuria. Over the years, numerous infections have complicated the course, particularly urinary tract infections. Therefore, repeated consideration has been undertaken whether lowering the level of immunosuppression would be possible. Since the transplant function is excellent, the patient refused to have a transplant biopsy. Screening for HLA antibodies repeatedly showed specificities in both classes I and II (Table 1). Since we do not have any HLA information on the donor, we could not define the donor-specificity of these antibodies.

Establishment of collecting donor DNA

As shown above, the inability to assign donor-specificity to HLA antibodies due to missing donor HLA information is a recurrent problem. In the two described cases, the donors lived abroad and there was no possibility to obtain the donor HLA-types or DNA. We therefore attempted to acquire donor DNA in order to perform retrospective HLA typing. One option was a transplant biopsy. This was obtained from Patient A as an independent/parallel biopsy to the diagnostic one depicted in Fig. 2a. The biopsy cylinder was immediately stored at −80 °C and DNA was extracted subsequently. Another option was expanding donor tubular cells from the recipients' urine as described earlier [15,16]. Human urinary Primary Tubular Cells (huPTC) were cultured over a period of 3 weeks, with two passages until a confluent 10 cm^2 culture dish was available for DNA extraction (Fig. 3a). The cells were purely epithelial

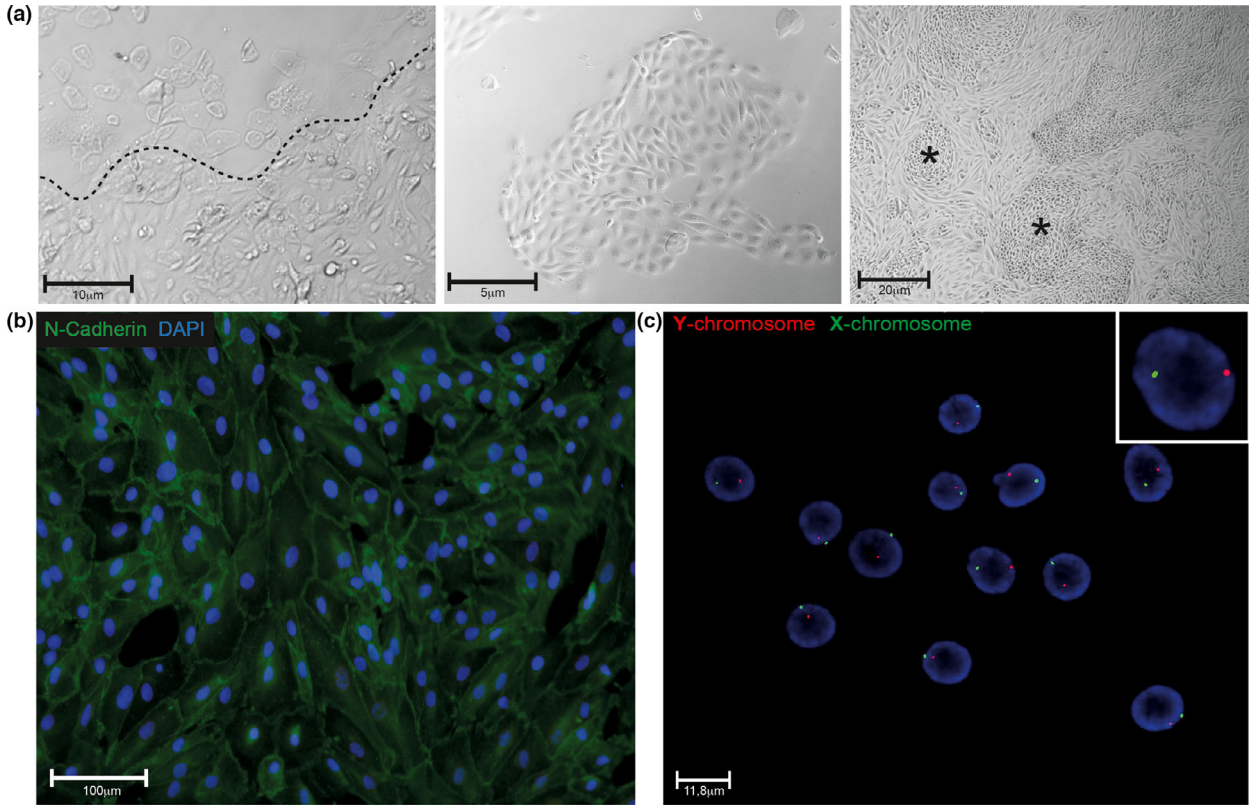

Figure 3 Establishment of donor derived human urinary Primary Tubular Cells (huPTC). Cells from a spontaneous urine sample were seeded into a 12-well culture plate, where initially floating squamous cells from the urothelium (above dotted line in left panel of a), as well as mostly adherent tubular cells can be seen (below dotted line in left panel of a). Over time (approximately 1–2 weeks) the squamous cells are lost and numerous growing colonies of huPTC can be detected (middle panel in a). After at least one round of trypsinization the cultures appear purely epithelial and form domes when confluent (asterixes, right hand panel in a). Immunostaining for renal tubular cells with N-cadherin show a complete layer of epithelial origin (b). Cell nuclei were stained with DAPI. The male donor huPTC from the urine of the female Patient A were grown on glass slides and analysed by FISH for X- and Y-chromosomes (c). All cells display one X-(green) and one Y-chromosome (red). Thus, all cultured cells were derived from the male donor. Scale bar indicates the magnification. A representative image of the stained nuclei of the male donor gender is displayed (further enlarged) in the top right corner of the panel.

from the first passage on, which was corroborated by staining for N-cadherin (Fig. 3b). Since the KTx of Patient A in 2013 was a male into female transplantation, donor origin of the cells was easily confirmed by fluorescence in situ hybridization (FISH) analysis with markers against the X and Y chromosomes. After three passages, FISH clearly identified 100% of cultured huPTCs to be of male origin (Fig. 3c). To further substantiate purity and origin at the DNA level short tandem repeat (STR) analysis was performed. Figure 4 shows representative results of STR analysis using DNA from the Patient A, her graft biopsy and the donor huPTC. The STR profiles allowed for the clear distinction of recipient and donor huPTCs DNA, whereas DNA from the graft biopsy displayed a mixture of approximately 50% of both genetic backgrounds. Importantly, this cross-contamination of donor and recipient DNA in the biopsy effectively prevented further analysis.

We performed HLA typing from peripheral blood of the Patient A, her spouse and the donor huPTC. Table 1 shows the detailed results of the Luminex analysis of HLA antibodies and the HLA typing of each individual material, clearly identifying HLA-DQ2 and -DQA1*05 as DSA. Interestingly, the spouse of our patient also carries these two antigens. It is therefore tempting to speculate that the two failed pregnancies 2017 (in conjunction with the reduced immunosuppression) may have led to immunization resulting in AMR. To our knowledge, the patient has not attempted to become pregnant before 2017. Figure 5 schematically summarizes these data sets.

For Patient B, retrospective donor typing from huPTC was also successfully carried out (Table 1). This

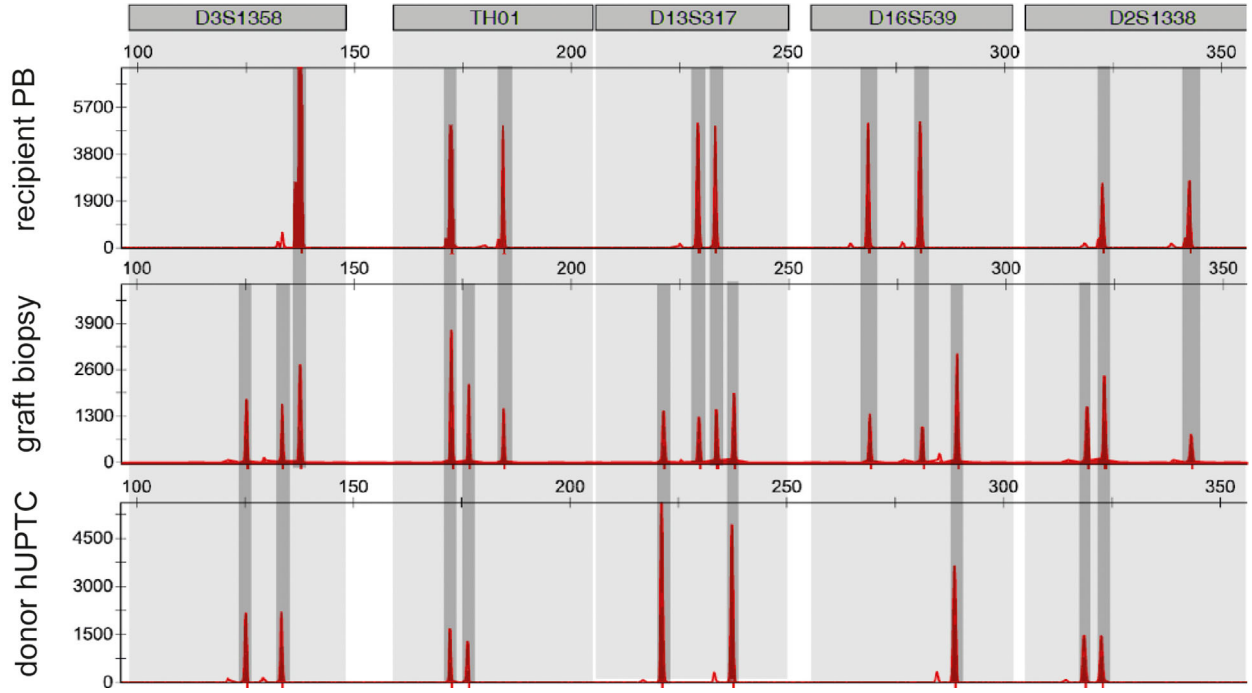

Figure 4 Purity of investigated DNA. The DNA of the peripheral blood (PB) from Patient A, the graft biopsy and the huPTC derived from the donor were analysed by short tandem repeat (STR) analysis. Representative results are shown for selected polymorphic markers, as detailed in the top row. The signals clearly show that the DNA of Patient A and the huPTC clearly stem from distinct individuals, whereas the DNA extracted from the graft biopsy appears to be derived roughly in equal parts from both these individuals. The intensity of the signals is indicated as relative fluorescence units (RFU) on the left hand side of each panel.

allowed for the recognition of low-level antibodies against HLA-DR11, and −16, as well as, HLA-DR52 as donor specific. In contrast to case A, all antibodies had MFIs below 1000, possibly in line with an overall stable function of the allograft.

Validation of urinary cell cultures in further 10 patients

To further assess whether huPTCs are a viable diagnostic option to retrieve donor DNA in a noninvasive manner, we expanded our case study by analysing huPTC samples from 10 additional KTx patients. The patients were randomly selected from current routine clinical care patients for which DNA samples from peripheral blood of living or postmortal donors were retained from the time to transplantation. Table 2 lists the clinical characteristics of all patients investigated.

We successfully established huPTC cultures from the urine of all 10 patients. Although two individual samples initially did not yield proliferating cells, huPTCs could still be established after repeated sampling. Once the surface of the initial 12-well plate exceeded 60% of cells, trypsinization to a 6-well plate was performed. Depending on the proliferation rates of individual samples, the culture to a confluent 10 cm^2 culture dish for DNA extraction took 2–4 weeks.

Conceivably, residual urine production before KTx could lead to shedding also of recipient tubular cells. Those may potentially contribute to a mixed recipient/donor huPTC culture after KTx. Indeed, we did observe one case (patient no. 5, Table 2) where 3.8% recipient derived huPTCs in the culture were detected by FISH (Fig. 6). Interestingly, this patient was sampled four weeks after KTx and had approximately 200 ml of residual daily urine production. With this single exception, all other samples were of pure donor origin, as measured by STR analysis (Fig. 7). Accordingly, we successfully determined the complete donor HLA types from huPTC-derived DNA in all tested cases. Importantly, the huPTC-derived HLA-type was identical to that derived from the stored donor DNA (Table S1), with the exception of patient no. 5, where recipient huPTC were also cultured in small amounts (Table 2, Fig. 6).

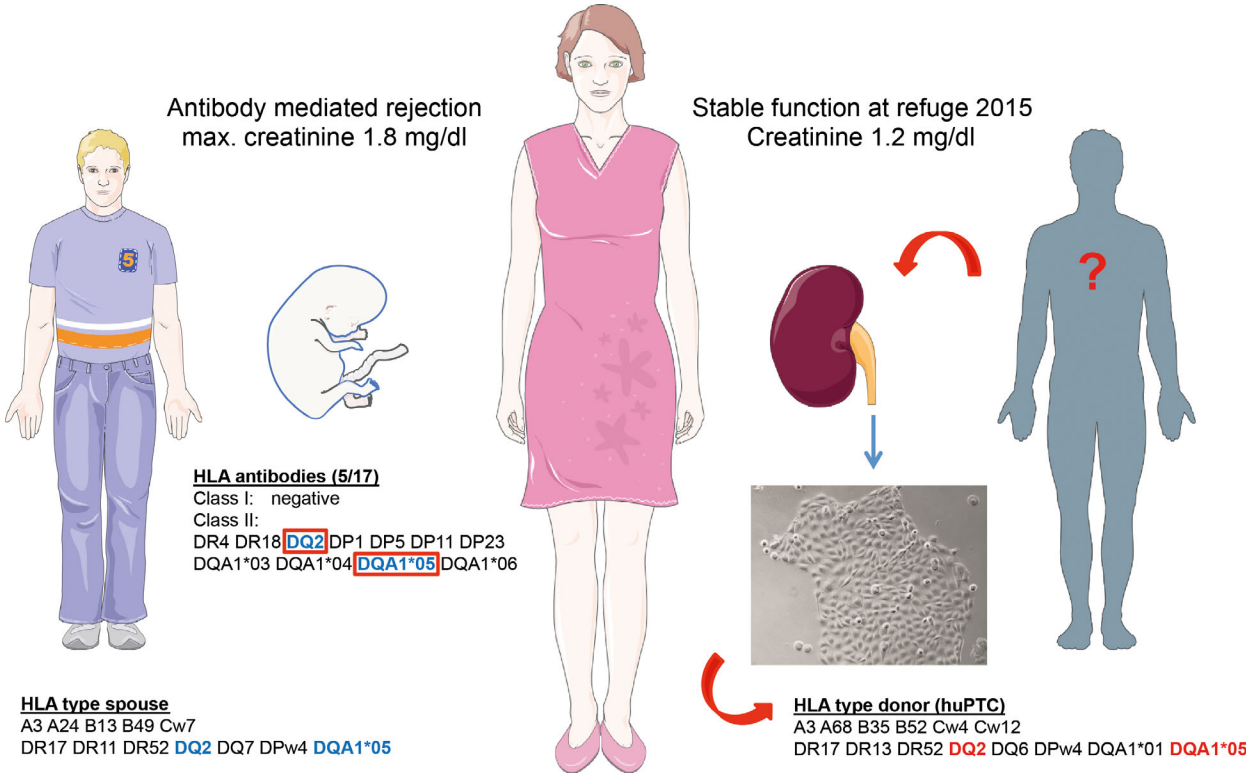

Figure 5 Scheme of events and findings for Case A. Patient A received a KTx by life kidney donation in Iraq in 2013, with excellent initial transplant function. After her refuge to Germany, she experienced two pregnancies, which led to AMR with KTx deterioration and positivity of class II antibodies. Since no HLA information of the kidney donor was available, the specificity of the antibodies could not be assigned. The HLA types of the spouse and Patient A herself (not shown) were determined by DNA extracted from peripheral blood. The respective donor information was gathered by culturing donor derived huPTC from the recipient's urine. Antibodies against DQ2 and DQA1*05 were donor specific, which in 2017 showed a peak MFI >20 000 (see Fig. 1). These specificities can be found in the HLA types of the spouse (blue), as well as the kidney donor (red).

Table 2. Clinical data.

Patient ID	Age recipient, years	Δ Time, months	Donor sex	Recipient sex	Donation modality	Positive control
Case A	31	70	M	F	Living donation	None
Case B	40	131	M	F	Living donation	None
1	58	1	M	M	Postmortal	Donor leukocytes
2	65	1	M	M	Postmortal	Donor leukocytes
3	70	10	M	M	Postmortal	Donor leukocytes
4	59	6	M	F	Postmortal	Donor leukocytes
5	41	1	F	M	Postmortal	Donor leukocytes
6	36	1	F	M	Postmortal	Donor leukocytes
7	60	1	F	M	Living donation	Donor leukocytes
8	46	50	M	F	Postmortal	Donor leukocytes
9	20	1	F	M	Living donation	Donor leukocytes
10	51	94	M	F	Postmortal	Donor leukocytes

Discussion

Avoiding and/or treating AMR currently appears to be the most important challenge in enabling long-term KTx [7,12]. Diagnosing and monitoring AMR, however, strongly depends on the early detection of DSA. Therefore, incomplete donor HLA types represent a clinically relevant diagnostic challenge because solid-phase antibody testing is regularly lacking the necessary information to assess donor specificity of the detected

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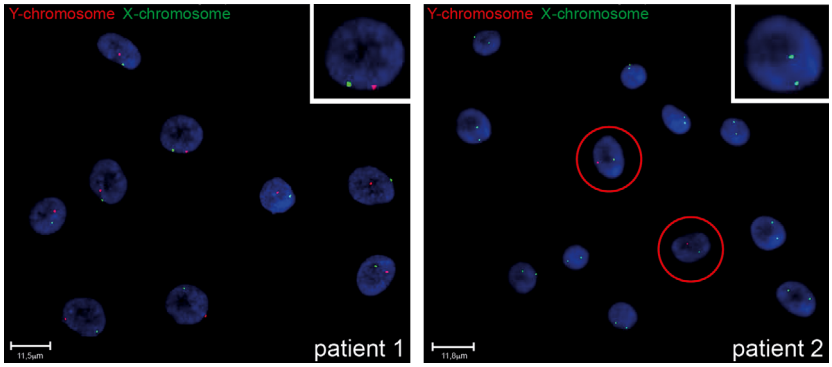

Figure 6 Origin of huPTC differentiated by gender. The origin of cultivated cells was determined by FISH for X- and Y-chromosomes on huPTC grown on glass slides. The female Patient B received a KTx from a male donor 131 months before this analysis (see Table 2). FISH shows that 100% of the huPTC are derived from the male donor, displaying the male genotype (left hand panel). Patient no. 5 received a postmortal KTx from a female donor, only one month before this analysis (see Table 2), with remaining diuresis of approximately 200 ml/day. FISH shows that most huPTC display the female, donor genotype (right hand panel). However, a small percentage of 3.8% cells are clearly of male gender (red circle). Scale bar indicates the magnification. A representative image of the stained nuclei of the respective donor gender is displayed (further enlarged) in the top right corner of each panel.

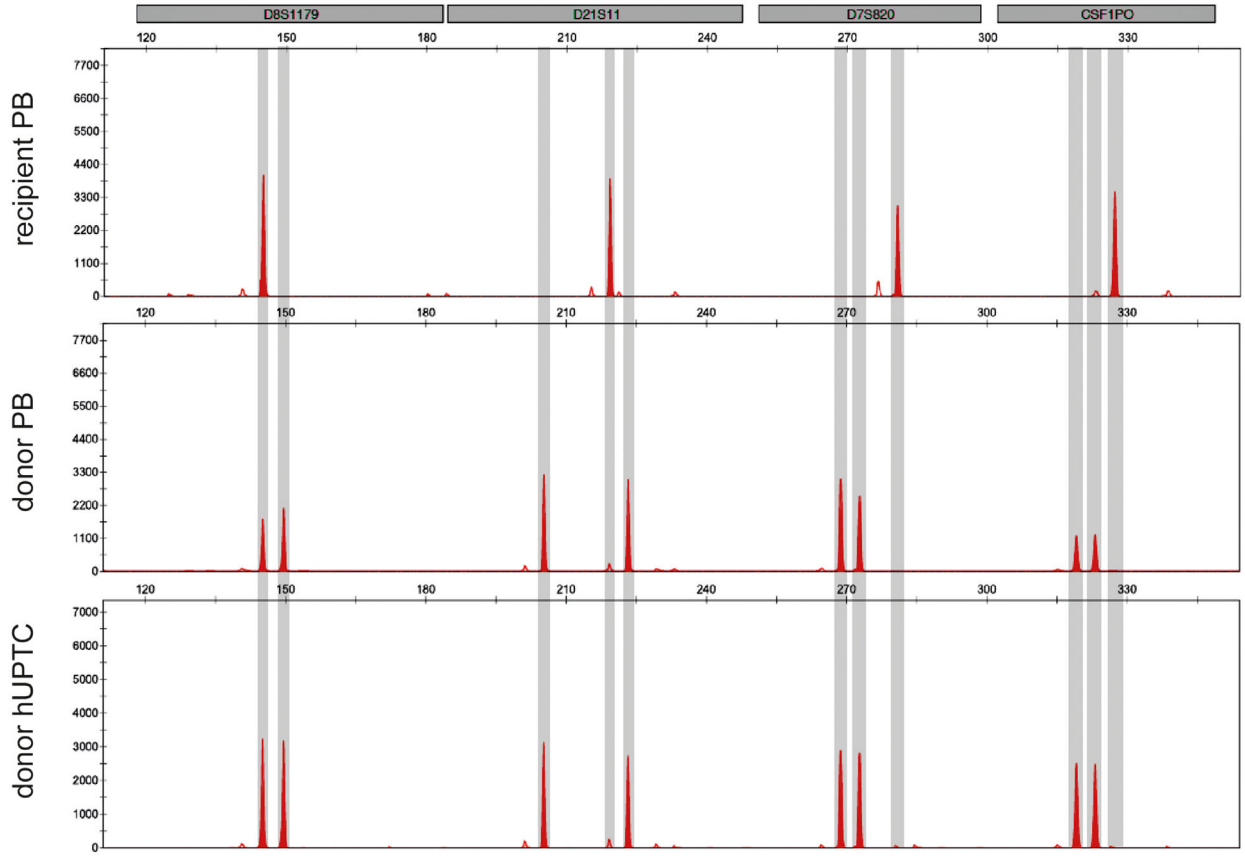

Figure 7 Donor origin of urinary cultures. A representative short tandem repeat (STR) analysis is displayed for the DNA of the peripheral blood (PB) from the recipient, the donor leukocytes and the huPTC derived from the donor. Representative results are shown for selected polymorphic markers, as detailed in the top row. The signals clearly show that the DNA of the recipient is distinct to the huPTC and donor leukocytes, where the latter two are identical. The intensity of the signals is indicated as relative fluorescence units (RFU) on the left hand side of each panel.

antibodies. We observe this problem in more than 20% of post-transplant testing, which is clearly underreported in the medical literature.

Our study demonstrates the feasibility of donor HLA typing from spontaneous patient urine samples collected any time after transplantation. We successfully established tubular cells from patient urine in all 12 investigated cases. This indicates broad applicability of this approach provided that rapid processing of urine samples is feasible. Importantly, we verified by FISH and STR analysis that primary tubular cells derived from recipient urine are a reliable source of donor DNA. Therefore, we regard this option a valuable, noninvasive new tool for kidney transplant diagnostics. Importantly, this tool may be sensible for clarification of HLA antibody specificities. It will not substitute the histopathological assessments of AMR in a transplant biopsy.

One option to acquire donor DNA if the donor is unavailable is from a transplant biopsy. However, due to the invasive nature of the procedure serious complications may arise, in rare cases even resulting in the loss of the transplant. Furthermore, from our experience (this study and [15]) many standard transplant biopsies deliver very little DNA and are frequently cross-contaminated with recipient tissue, which may be perirenal fat or recipient derived blood and immune cells. Based on STR analysis, this 'contamination' of donor with recipient DNA can reach approximately 50% (Fig. 4). This effectively precludes HLA typing with many standard techniques which rely on the presence of a maximum of two different HLA-alleles per locus to resolve typing ambiguities. More extensive studies are certainly warranted. However, the data from our limited data set supports the hypothesis that huPTCs from patient urine samples can be a superior source of donor DNA.

Although we demonstrated that urinary cells are both practical and reliable sources for donor DNA, there are some putative technical challenges associated with this approach. Firstly, the current protocol requires a tissue culture set-up in close vicinity to the clinical area, since prolonged storage of the urine does not produce viable cell cultures. Secondly, the culture of sufficient cells for DNA extraction will take a minimum of two weeks, precluding the immediate analysis for clinical purposes. Thirdly, despite the presence of antibiotics and antimycotics in the culture medium sporadic microbial contamination can occur. Interestingly, in rare instances urine samples do not produce viable cells for reasons other than the above, which has been described before [14]. However, in the latter cases educating the patient of best practices in sampling and repeat sampling did frequently resolve the problem. Finally, residual recipient diuresis at the time of transplantation can result in a mixed culture of recipient and donor tubular cells (Fig. 6). Conceivably, contribution of recipient cells to the culture will decrease over time, as diuresis of the native patients' kidneys will gradually decrease once the transplant establishes its function. Nevertheless, STR analysis or similar techniques can be used to confirm the donor origin of the cultured DNA (Figs. 3 and 7).

The two cases presented here in detail, exemplify the need for alternative methods to acquire donor DNA for patients currently under active clinical care. Ideally, complete HLA tissue typing information, extracted DNA or tissue of all transplant donors remains accessible for clinicians for follow-up diagnostic purposes. This, however, is impractical, in particular at the global scale. Moreover, which loci at which level of resolution constitute complete HLA typing information is actively being revised. HLA matching of kidney donors and recipients has evolved from matching serologically defined HLA-A, -B and -DR antigens, to matching molecularly defined HLA-A, -B, -C, -DR and -DQ antigens. More recently, several studies indicated that HLA-DP and also HLA-DQA mismatches are profoundly associated with adverse outcomes, prompting active debate about the inclusion of HLA-DP and HLA-DQA for HLA matching [17–20].

Another currently ongoing development is switching from traditional antigen-based towards epitope-based matching which potentially provides huge benefits for the matching process (e.g. reviewed in Ref. [21]). The benefits may include a larger pool of potential zero mismatch donors, avoidance of highly immunogenic donor/recipient constellations, and definition of permissive mismatches which may all contribute to an overall improved outcome [22]. However, this approach requires allele-level resolution of all major HLA-antigens for both donor and recipient. Therefore, new matching strategies and improved typing and antibody detection techniques constantly evolve the definition of antibody donor-specificity which may require retyping of donors. Taken together, this underlines the need for reliable sources of donor DNA for future-proof state-of-the-art follow-up monitoring of transplant patients.

As demonstrated here, donor huPTCs derived from recipient urine are a noninvasive, reliable source of donor DNA for retrospective donor HLA typing. We, therefore, consider them a valuable addition to the repertoire of kidney transplant physicians, filling current diagnostic gaps and potentially helping to meet future HLA typing requirements for state-of-the-art clinical care.

Authorship

CB, KXK and MH: performed experiments and analysed data. MK: performed FISH analyses. FP and KA: performed biopsy stainings. MSt and KS: assisted in performing experiments. DZ, KL and AD: provided data from the transplant centres. MSc and AR: interpreted and discussed the data. MSW and BMS: supervised the project, analysed data and wrote the manuscript.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. HLA typing data are shown for the 10 kidney recipients of the confirmation cohort, including donor typing and typing of the huPTC.

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