

## ORIGINAL ARTICLE

# Decreased mineralocorticoid receptor expression in blood cells of kidney transplant recipients undergoing immunosuppressive treatment: cost efficient determination by quantitative PCR

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**Aims:** Electrolyte imbalances caused by impaired ion transport are a frequent side effect of immunosuppressive treatment in renal transplant recipients. Clinical symptoms resemble features of hypoaldosteronism, although concentrations of aldosterone are in the normal range. Because immunosuppression might affect the hormone receptor status of cells, mineralocorticoid receptor (hMR) expression by peripheral blood leucocytes (PBL) was studied in these patients.

**Methods:** Twenty one renal transplant recipients being treated with cyclosporine A and 19 healthy controls were tested. hMR expression was quantified by means of competitive reverse transcription polymerase chain reaction (cRT-PCR) and compared with receptor binding studies with subsequent Scatchard plot analysis carried out previously on 20 renal transplant recipients and 25 controls. Advantages of PCR were summarised and compared with Scatchard plot analysis.

**Results:** Cyclosporine A caused a 37% decrease in hMR molecules on PBL in 75% of renal transplant recipients, and this effect was attributable to the downregulation of hMR transcription. PCR was 99% specific for the detection of hMR in PBL and highly reproducible.

**Conclusions:** Decreases in hMR protein and RNA in PBL of transplant recipients revealed an inhibitory effect of cyclosporine A on hMR transcription. Because hMR acts as a transcription factor, the expression of several genes involved in electrolyte homeostasis is affected, leading to signs of nephrotoxicity that require therapeutic adjustments. Because of the small volume of blood, the assay can be repeated during treatment and is therefore useful for measuring treatment outcomes. Lower costs and the absence of radioactive challenge are further advantages of the PCR method.

Transplant recipients under immunosuppressive treatment with calcineurin inhibitors (for example, cyclosporine A or FK 506) frequently show electrolyte alterations, such as hyperkalaemia and metabolic acidosis.<sup>1</sup> These symptoms clinically resemble features of hypoaldosteronism, despite normal plasma aldosterone concentrations in these patients. Therefore, we hypothesised that cyclosporine A and FK 506 are able to induce mineralocorticoid resistance of the distal tubule, leading to electrolyte imbalances in transplant recipients. Aldosterone is a key hormone involved in the regulation of electrolyte homeostasis through ion transport stimulation. It classically acts via the mineralocorticoid (aldosterone) receptor (hMR) on the epithelium of the kidneys, colon, and sweat glands to maintain electrolyte homeostasis.

"We established a polymerase chain reaction protocol that permitted the quantification of aldosterone receptor expression in both renal transplant recipients and healthy controls"

In the past, the density of aldosterone receptors has been determined by radioreceptor assays, but this method is limited to renal transplant recipients without evidence of anaemia, because 400 ml of blood is needed for each analysis. Furthermore, it does not permit the investigation of the influence of cyclosporine A on aldosterone receptor gene expression. Therefore, we established a polymerase chain reaction (PCR) protocol that permitted the quantification of

aldosterone receptor expression in both renal transplant recipients and healthy controls. Competitive reverse transcription PCR (cRT-PCR) is an increasingly used method for quantifying specific mRNAs.<sup>2</sup> The technique involves co-amplification from test RNA with an internal standard using common primers in a single reaction. The standard competes for primers and enzyme and is therefore referred to as a competitor. Common applications of this assay are the expression studies of acute phase proteins<sup>3</sup> and cytokines<sup>4,5</sup> and monitoring infection with hepatitis C virus.<sup>6</sup> The quantitative readout from this cRT-PCR method correlates well with that obtained by realtime PCR<sup>7,8</sup> or by northern blotting, and it is at least 100 times more sensitive.<sup>3</sup> In our present study, we used both methods—a radioreceptor assay and cRT-PCR—to compare the number of receptor binding sites with the number of expressed RNA molecules, to determine the aldosterone resistance on the basis of receptor status.

## MATERIALS AND METHODS

### Preparation of samples

Blood samples from renal transplant recipients undergoing treatment with cyclosporine A (mean steady state concentrations, 120–150 ng/ml) and healthy controls were processed

**Abbreviations:** cRT-PCR, competitive reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hMR, human mineralocorticoid receptor

within two hours. Lymphocytes from 20 renal transplant recipients and 25 healthy volunteers were isolated from 400 ml of blood for the aldosterone binding assays and subjected to subsequent Scatchard plot analysis as described by Shipman *et al.*<sup>9</sup> For PCR analysis, 14 ml of blood was taken from 19 patients and 21 healthy volunteers. For PCR analysis, total RNA from 14 ml aliquots for each patient was isolated after hypotonic lysis of contaminating red blood cells using Trizol reagent (Gibco, Karlsruhe, Germany) and subsequent chloroform extraction. The remaining DNA was digested with 0.5 U/ $\mu$ l DNase I (Roche, Mannheim, Germany) for 30 minutes at 37°C. After phenol extraction and isopropanol precipitation, the RNA was dissolved in 50  $\mu$ l RNA storage buffer (Ambion-Europe, Huntington, Cambridgeshire, UK), and 0.4 U/ $\mu$ l RNase inhibitor (Roche) was added. The amount and purity of the isolated RNA was determined by photometry ( $A_{260}/A_{280}$ ). Aliquots of the RNA were stored at -20°C.

### Preparation of the hMR internal competitor<sup>5</sup>

The 472 bp hMR PCR product, amplified by the hMR sense and antisense primers from total RNA of a healthy volunteer (fig 1) in 34 cycles, was cleaved by the EcoRI restriction enzyme, yielding a 113 bp fragment and a 359 bp fragment, which were cut out of the gel and purified with the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Four step PCR amplification (fig 1) generated a 373 bp hMR product fused to the T7 promoter sequence, which permitted in vitro transcription in the T7 RiboMAX large scale RNA production system (Promega, Mannheim, Germany) for four hours at 37°C, according to the manufacturer's protocol. The amount of competitor RNA was determined by photometry and the RNA was frozen at -70°C in aliquots of  $10^9$  molecules/ $\mu$ l.

### cRT-PCR

Aliquots (0.5  $\mu$ g) of RNA were subjected to reverse transcription in the presence of decreasing concentrations of competitor RNA molecules (usually  $0.5 \times 10^2$ - $10^3$ ) using a first strand cDNA synthesis kit (Roche) and 40pM hMR antisense primer. Subsequently, 30  $\mu$ l of PCR mix (Gibco) and 40pM hMR sense primer were added to individual test tubes, which were then subjected to PCR for 38 cycles in a Personal cycler (Biometra, Göttingen, Germany). Cycling conditions consisted of 30 seconds of denaturation at 94°C, 30 seconds of annealing at 60°C, and 30 seconds of extension at 72°C. The primers recognised a 472 bp sequence unique to the coding region of the hMR gene (Genebank accession number

16801) and the 373 bp competitor RNA. Because of the narrow range of around  $10^2$ - $10^3$  expressed hMR molecules, the patients' RNA was also amplified with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (sense, 5'-ACC ACA GTC CAT GCC ATC AC-3'; antisense, 5'-TCC ACC ACC CTG TTG CTG TA-3') in the presence of GAPDH competitor RNA (Situs-Chemicals, Düsseldorf, Germany), yielding a 416 bp fragment from the patients' RNA and a 386 bp fragment from the competitor RNA. The housekeeping gene GAPDH is constitutively expressed and is therefore well suited to correct for differences in the amount of RNA subjected to cRT-PCR analysis. Following RT-PCR, 5  $\mu$ l aliquots of the PCR probes were subjected to electrophoresis in a 2% Metaphor agarose gel (Biozym, Hessisch-Oldendorf, Germany) for 2 hours at 100 V. After electrophoresis, the relative intensities of the ethidium bromide stained hMR and GAPDH DNA bands were evaluated by densitometry (Raytest, Straubenhardt, Germany). The number of hMR molecules was calculated by linear regression analysis of the logarithm of copies of RNA competitor versus the log ratio of hMR  $RNA_{patient}/hMR RNA_{competitor}$  and corrected for the definite number of  $1 \times 10^5$  GAPDH molecules.

### Sequencing

The 472 bp hMR and the 373 bp competitor fragments were sequenced once using an ABI 310 genetic analyser (Applied Biosystems, Foster City, California, USA). Sequence data analysis was performed with Sequencer 4.0.5 (Gene Codes, Ann Arbor, Michigan, USA) and BLASTN software (kindly provided by the genome project Japan) to ensure the identity and purity of the amplified fragments.

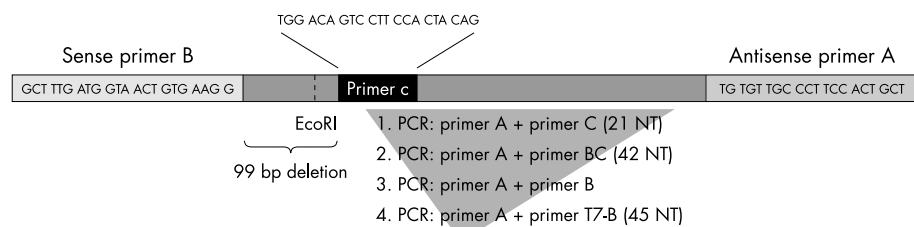
### Statistical analysis

Statistical analysis was performed using the Student's *t* test for unpaired comparisons, with  $p < 0.05$  being considered significant.

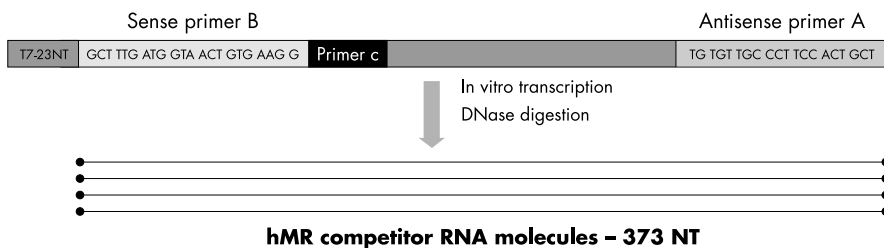
### RESULTS

The construction of a competitor hMR RNA enabled the expression level of hMR in human peripheral blood leucocytes to be measured by quantitative RT-PCR. Sequence analysis of the PCR products revealed exclusive amplification of hMR RNAs. Figure 2 shows the cRT-PCR products of one patient and the subsequent evaluation by linear regression analysis. In addition to the determination of hMR mRNA molecules in 0.5  $\mu$ g RNA, we correlated the

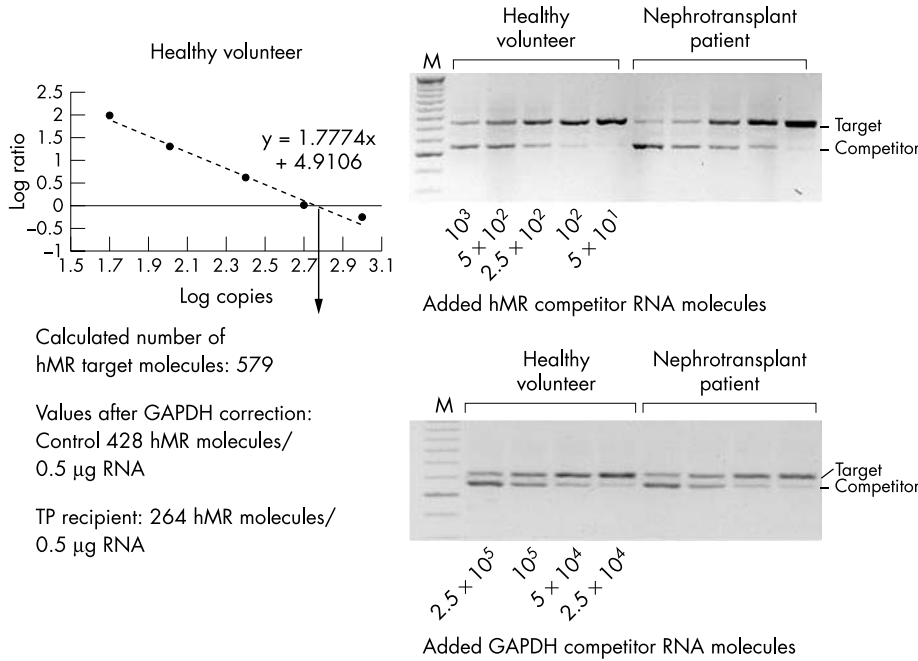
### hMR cDNA - 472 bp



### hMR competitor



**Figure 1** In vitro synthesis of the 373 bp internal mineralocorticoid receptor (hMR) competitor by reverse transcription polymerase chain reaction and subsequent transcription.



**Figure 2** Determination of expression of mineralocorticoid receptor (hMR) in human blood cells by competitor reverse transcription polymerase chain reaction with subsequent densitometry and evaluation. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TP, transplant.

number of receptor RNA molecules with  $1 \times 10^5$  GAPDH molecules, also determined by quantitative PCR. Because GAPDH is constitutively expressed in cells, this correlation corrects for differences in the photometric determination of RNA concentrations.

In our study, we analysed 40 volunteers by this method (fig 3A). The renal transplant recipients (n = 21) had significantly lower expression than the healthy control group (n = 19) ( $p < 0.0012$ ), although there was high individual variance. Longterm expression studies in healthy volunteers revealed that expression varied by a factor of four. Repeated CRT-PCRs using identical RNA probes after one to eight days of storage reflect the effect of storage and different enzyme batches on quantitative PCR analysis.

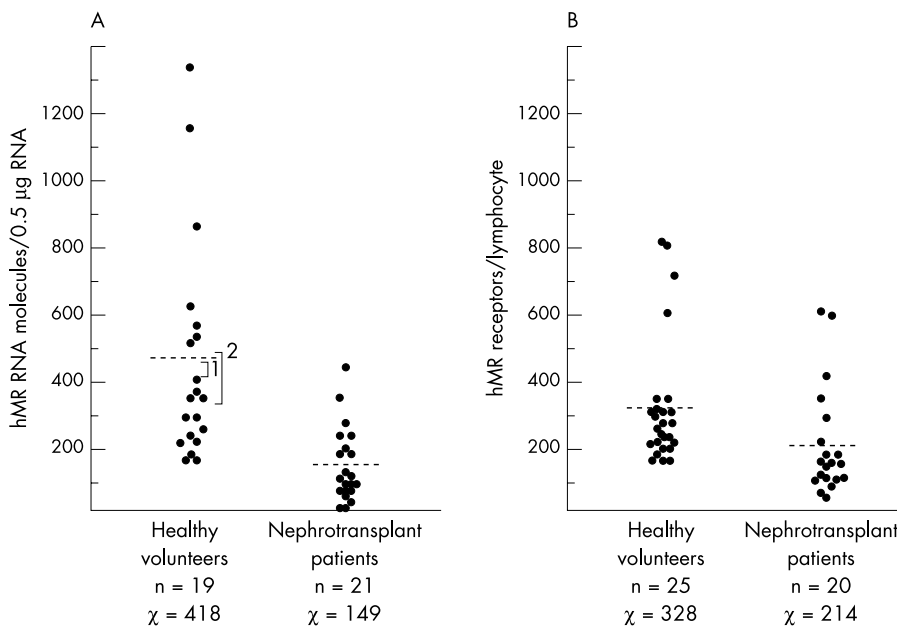
Figure 3B shows the results of the radioreceptor assays on a cohort of 45 volunteers, different from those tested in the CRT-PCR assay. These assays also revealed a decreased

number of receptor binding sites on the lymphocytes of graft recipients, but the difference between the healthy controls and the transplanted patients was not significant ( $p < 0.157$ ). Because of the large volume of blood needed for each analysis, the assays could not be repeated, and no standard deviations were determined.

Comparison of the results of the CRT-PCR (fig 3A) and the radioreceptor assay (fig 3B) confirmed the different distribution of hMR molecules in the healthy and transplanted groups, although the number of molecules/0.5 µg RNA in the PCR analysis cannot be directly compared with the number of protein molecules/lymphocyte.

**DISCUSSION**

Immunosuppressive treatment after solid organ transplantation leads to hyperkalaemia and metabolic acidosis, possibly as a result of aldosterone resistance in approximately 75% of



**Figure 3** Distribution of patients and healthy volunteers by the expression level of mineralocorticoid receptor (hMR) in their blood cells detected either by (A) quantitative reverse transcription polymerase chain reaction (qRT-PCR) or (B) conventional receptor binding studies. Median values are indicated for each group, and were significantly lower in patients undergoing immunosuppressive treatment, when investigated by qRT-PCR. The range of values labelled with "1" reflects the variation of hMR expression in a single RNA preparation as a result of storage for one to eight days and different enzyme batches analysed in four independent PCR reactions. The range of values labelled with "2" reflects the variance of hMR expression of one healthy volunteer in a longterm study covering two months.

	cRT-PCR	Scatchard plot
Blood volume	10 ml	400 ml
Patient's stress	No	High
Repetitions	Up to 4	No
Multiple assays in parallel	Up to 6	No
Time spent (hands on time)	2 days	1 day
Hazards	Phenol	Radioactivity
Costs/assay	44 Euros	167 Euros

**Figure 4** Comparison of the advantages and disadvantages of competitor reverse transcription polymerase chain reaction (cRT-PCR) and radioreceptor assay for the determination of mineralocorticoid receptor expression in blood cells.

patients. This effect is not limited to kidney cells. Armanini *et al* described the downregulation of mineralocorticoid and glucocorticoid receptors by lymphocytes in patients suffering from anorexia nervosa.<sup>10</sup> Glucocorticoid administration has also been shown to effect the number of glucocorticoid receptors on lymphocytes.<sup>9</sup> In our present study, we examined whether this resistance correlated with the reduced expression of aldosterone receptors on blood cells in renal transplant recipients. The determination of free receptor binding sites on lymphocytes revealed 214 receptor molecules/lymphocyte compared with 328 molecules/lymphocyte in healthy controls ( $p < 0.157$ ). This explains why symptoms of hypoaldosteronism are seen in transplant recipients despite normal aldosterone concentrations in the blood. Recently, Matsuda *et al* described the binding of cyclosporine A to immunophilins, which interact with the receptor in its non-functional status.<sup>11</sup> The formation of this complex might fix the receptor in the cytoplasm, thereby reducing the number of free receptor binding sites on the lymphocyte membrane. In addition, a decrease in the rate of transcription of the hMR gene could also explain the decreased number of hMRs on each lymphocyte. Indeed, further analysis by quantitative RT-PCR with hMR specific primers revealed decreased transcription of the gene in patients undergoing cyclosporine A treatment.

The mean number of hMR RNA molecules/0.5 µg RNA dropped from 468 molecules in untreated controls to 169 molecules in renal graft recipients ( $p < 0.002$ ). The fact that the reduction was greater at the transcriptional level than at the protein level suggests that translation rates might be increased to compensate for the missing RNA molecules.

“It would be useful to find immunosuppressive drugs without side effects on mineralocorticoid receptor expression”

Our study further showed that both methods (the determination of receptor binding sites and quantitative PCR) are equally suitable for detecting the reduced number of aldosterone receptors on blood cells. Nevertheless, quantitative PCR—at least in laboratories where PCR technology is established—has some advantages, as listed in fig 4. Goerke *et al* described it as a robust technique, which is not susceptible to traces of PCR inhibitors in contrast to LightCycler RT-PCR.<sup>8</sup> In particular, continuous control of hMR expression during the treatment of hyperkalaemia and

### Take home messages

- Decreased numbers of mineralocorticoid receptors (hMRs) were detected in 75% of patients who were undergoing immunosuppressive treatment with cyclosporine A after kidney transplantation, leading to hyperkalaemia and metabolic acidosis as a result of aldosterone resistance
- Both the measurement of hMRs/lymphocyte by the conventional radioreceptor assay and the measurement of specific RNA by competitor reverse transcription polymerase chain reaction (cRT-PCR) were suitable methods for detecting these decreases
- Because of the small volume of blood needed for the newly developed cRT-PCR, the assay can be used to monitor treatment outcomes. Lower costs and the absence of radioactive challenge are further advantages of the PCR method

metabolic acidosis is possible only with quantitative PCR, and it could be useful in the search for drugs that not only repair ion transport in the renal tube but also increase receptor expression on the cells. Furthermore, it would be useful to find immunosuppressive drugs without side effects on hMR expression.

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