Clinical research

Cardiac angiotensin II receptors as predictors of transplant coronary artery disease following heart transplantation


Aims We tested the hypothesis that cardiac angiotensin II (Ang II) receptor gene transcription may predict the development of transplant coronary artery disease (TCAD) following heart transplantation.

Methods and results We examined the gene transcripts of Ang II type 1 (AT1R) and type 2 receptors (AT2R) in endomyocardial biopsy specimens from 50 heart transplant recipients. The progression of TCAD was measured as change in maximal intimal thickness (CMIT) and change in plaque volume (CPV) by intravascular ultrasound (IVUS) examinations from baseline to one year after transplantation. The level of AT1R mRNA was associated with that of AT 2R in transplanted hearts (regression coefficient $\beta = 1.77$, 95% CI 0.85–2.89, $P < 0.0001$). AT1R and AT2R gene transcripts were univariate predictors of CMIT (AT1R: regression coefficient 0.10, 95% CI 0.06–0.14, $P < 0.0001$; AT2R: regression coefficient 0.28, 95% CI 0.17–0.40, $P < 0.0001$) or CPV (AT1R: regression coefficient 0.41, 95% CI 0.17–0.65, $P < 0.0001$; AT2R: regression coefficient 1.25, 95% CI 0.49–2.01, $P = 0.002$). By one year, 21 (46%) transplant recipients showed evidence of transplant vasculopathy and the rest did not. The vasculopathic group demonstrated a higher level of expression of cardiac AT1R than the non-vasculopathic group (3.7 ± 2.9 vs 1.6 ± 1.7 folds; $P = 0.006$). The level of AT1R mRNA in transplanted heart was identified as a discriminator that predicted the development of transplant vasculopathy with a sensitivity of 75% and specificity of 83%.

Conclusions Cardiac Ang II receptor gene transcripts are associated with the progression of TCAD following heart transplantation. Only AT1R gene transcripts predicted the development of transplant vasculopathy in this preliminary study.

KEYWORDS
Transplant coronary artery disease;
Angiotensin II receptors;
Intravascular ultrasound

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Introduction

Transplant coronary artery disease (TCAD), which affects 44% of transplant recipients by 3 years as assessed by coronary angiography, is a major limitation for the long-term success of cardiac transplantation. However, intravascular ultrasound (IVUS) has detected abnormal intimal thickness in 50% of patients one year after cardiac transplantation. Although considerable progress has been made in the diagnosis of TCAD, many questions remain concerning its pathogenesis. Both immune and non-immune mechanisms have been implicated in the progression of TCAD. Earlier, we described an association between ischaemic cardiac injury or cardiac vitronectin receptor expression and the development of TCAD, which suggested the importance of non-immunological factors in the pathogenesis of TCAD. More recently, the role of the tissue renin-angiotensin system (RAS) in the pathogenesis of native coronary artery disease has been well documented. Now it is generally recognized that locally produced angiotensin II (Ang II) activates the cells regulating the expression of a host of biological mediators, including growth factors, cytokines, chemokines, and adhesion molecules involved in the pathogenesis of conventional atherosclerosis and thus could play an important role in the pathogenesis of TCAD as well.

Consistent with this concept, a few unconfirmed reports have implicated RAS in the pathophysiology of TCAD. Furthermore, risk factors such as hypertension and hypercholesterolaemia, which increase the probability of TCAD following transplantation, are also associated with activation of RAS. Ang II is the final effector molecule of the RAS and mediates its biological actions mainly through two types of receptors — Ang II type 1 (AT1R) and type 2 (AT2R).

On the basis of these data, we hypothesized that heart transplant recipients with TCAD would have increased levels of cardiac Ang II receptor gene transcripts and that these levels might be associated with the progression of TCAD. To examine this hypothesis, we investigated the levels of AT1R and AT2R gene transcripts in transplanted hearts to determine their predictive value for the progression of TCAD, measured as change in maximal intimal thickening (CMIT) and change in plaque volume (CPV) by IVUS in heart transplant recipients.

Methods

Study patients

Fifty heart transplant recipients underwent surveillance right ventricular endomyocardial biopsies and intravascular ultrasound (IVUS) studies. Endomyocardial biopsies obtained throughout the first year post-transplantation were processed for histological examination to assess possible cellular rejection and to determine average biopsy score (ABS). A portion of the biopsy sample obtained one year after transplantation was snap-frozen for subsequent analysis for AT1R and AT2R mRNA transcripts. Coronary angiography and IVUS were performed at baseline (within 4 weeks) and one year after heart transplantation to assess TCAD. The protocol was approved by the Institutional Review Board of the Cleveland Clinic Foundation.

Intravascular ultrasound

The intravascular ultrasound technique has been reported in detail. Briefly, using a standard technique for intracoronary catheter delivery, the operator advances the imaging device into the transplant coronary artery to the most distal position that can be safely reached and then retracts it at a constant speed using an automated pull-back system while recording serial cross-sectional images of the artery on a super VHS video tape for subsequent analyses. Proximal, mid and distal segments of the three major epicardial coronary arteries, defined according to the Coronary Artery Surgery Study classification, were targeted for imaging. The images were analysed in the IVUS core laboratory by an investigator who was blinded for the study. Anatomical landmarks were used to identify identical segments for serial examinations. Matched sites were analysed at baseline (within 4 weeks) and one year after transplantation and the following measurements were made. Fig. 1 displays the IVUS images of the transplant coronary artery obtained at 4 weeks and one year after transplantation.

Maximal intimal thickness at most affected site: The greatest distance from the intimal leading edge to the leading edge of the adventitial border.

Plaque volume: The cross-sectional area (Fig. 1) was measured as the difference between vessel and lumen area. Plaque volume was derived from the measurements of maximal intimal thickness at two consecutive points along the length of the most affected site.

Change in maximal intimal thickness (CMIT): The difference in maximal intimal thickness between the one-year and baseline measurements.

Change in plaque volume (CPV): The difference in plaque volume between one year and baseline.
Transplant vasculopathy: The threshold to define transplant vasculopathy was CMIT $\geq 0.3$ mm over one year, based on earlier reports of intimal thickening that could be considered pathological.\(^{14}\)

Endomyocardial biopsies

Using a standard transjugular approach, a series of right ventricular endomyocardial biopsies were made as part of the surveillance program for early detection of rejection. The endomyocardial tissues were divided to parallel parts for histological analysis and RNA isolation. Specimens for histological analysis were fixed in formalin, routinely processed, and embedded in paraffin. Sections were cut and stained with haematoxylin and eosin to determine the grade of cellular rejection in accordance with the criteria for histopathological diagnosis of rejection established by the Working Formulation of the International Society of Heart and Lung Transplantation.\(^{15}\) The recipients of heart transplants underwent approximately 13 endomyocardial biopsies in the course of the first year post-transplantation. Each biopsy was assigned a score (1–6) depending on the degree of cellular rejection. The average biopsy score was calculated as the sum of the individual scores divided by the number of biopsies. Patients were classified into low (biopsy score $<1.0$), intermediate (biopsy score $1.0–1.5$), and high (biopsy score $>1.5$) biopsy score categories. The tissue destined for RNA isolation was immediately frozen in liquid nitrogen and stored at $-80$ °C.

Isolation of total RNA

Endomyocardial specimens were retrieved from frozen blocks (−80 °C) and rapidly processed to isolate total RNA using Totally RNA kit (Ambion Inc., Austin, TX) following the manufacturer's instructions. Briefly, 200 µl of denaturation solution was added and tissue was homogenized in a 1.5-ml nuclease-free tube. An equal volume of phenol:chloroform:IAA was added and the tube was agitated in a vortex and then stored on ice for 5 min. After centrifugation, the aqueous phase was transferred to a new tube and 1/10 volume of sodium acetate solution was added. An equal volume of acid:chloroform was added, then the tube was agitated in a vortex and then stored on ice for 5 min. After centrifugation, the aqueous phase was transferred to a new tube and tissue was homogenized in a 1.5-ml nuclease-free tube. An equal volume of phenol:chloroform:IAA was added and the tube was agitated in a vortex and then stored on ice for 5 min. After centrifugation, the aqueous phase was transferred to a new tube and precipitated with isopropanol and stored at $-20$ °C for 30 min. The precipitation mixture was centrifuged and supernatant was discarded. The pellet was washed with 70% ethanol and re-suspended in 50 µl diethyl pyrocarbonate-treated distilled water.

Assessment of RNA yield

The concentration and purity of RNA was determined by its absorbance in spectrophotometer at 260 nm using a bioanalyzer. During initial experiments, a portion of recovered total RNA was electrophoresed on a denaturing agarose gel to determine its purity.

Reverse transcription

RNA samples were reverse transcribed using the TaqMan reverse transcription kit (TaqMan Reverse Transcription Reagents, Applied Biosystems, Foster City, CA, USA). Aliquots of the mix were deposited in individual tubes and template RNA was added. Samples were incubated for 90 min at 25 °C, 45 min at 48 °C, and 5 min at 95 °C. A duplicate tube with no reverse transcriptase was included to control for DNA contamination.

Quantitative real-time polymerase chain reaction (TaqMan system)

AT$_R$ and AT$_2R$ primers and probes for quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) were designed using the PRIMER express program of Applied Biosystems. BLAST search was conducted against dbEST and nr (GenBank, EMBL) to confirm the total gene specificity of the nucleotide sequences and absence of DNA polymorphism. The oligonucleotide sequences of the TaqMan probe and primers were as follows: AT$_R$ – TaqMan probe FAM-1422 ATCC ACCAAAGCCTGACACCATGTT-TAMRA, forward primer 1350 AGGCCAATCCCCACTACAAACT, reverse primer 1470 TCAGAGAT GTCAACTCAACCTCA; AT$_2R$ – TaqMan probe FAM 948 CTGG CTTCTCATATTGTGCTCTCC-TAMRA; forward primer 919 AAG TCTCTAAGATGCGCAGC; reverse primer 1055 CAGGTCA TGACTGCTATAACTTG. Primers and probes were purchased from Perkin–Elmer, Applied Biosystems (Foster City, CA).

To measure gene expression, a reaction mix was prepared on ice with $10 \times$ TaqMan Buffer; 5.5 mM MgCl$_2$; 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 200 µM dUTP; Amylase UNG (0.01 U/µl), and AmpliTaq Gold DNA polymerase (0.025 U/µl), 185 forward ribosome, reverse primers and probe (all at 50 nM), forward and reverse primers for AT$_R$ and probe at 100 nM concentrations. Aliquots of the reaction mix were deposited in separate tubes of a 96-well plate and cDNA was added. The RT-PCR reaction was performed in a final volume of 50 µl with duplicates for each data point using an ABI Prism 7700 (Applied Biosystems). Each RT-PCR run included a no template control, no amplification control, the calibration standard, and unknown patient cDNA. The thermal cycling conditions comprised an initial denaturation at 50 °C for 2 min and at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 62 °C for 1 min. The data were analyzed using Sequence Detector Version 1.7 (Applied Biosystems). We used normal human heart total RNA for calibration. Using the 2$^{-\Delta\Delta CT}$ method of relative quantification, we reported the fold change in gene expression normalized to 18S ribosomal RNA and relative to 16.

Data analysis

Data were expressed as means with standard deviation. The two-tailed Student t-test, Mann–Whitney, and Kruskall–Wallis tests were used as appropriate to compare subgroups. Categorical variables were compared by Fisher’s exact test. The linear regression model was used to estimate the linear equations between gene transcripts of two types of receptor and some biochemical markers. The logistic regression model was constructed to assess the effects of cardiac levels of AT$_R$ and AT$_2R$ gene transcripts on the development of transplant vasculopathy. The assumption of linearity was tested with logistic regression by doing the following transformations on the linear regression model was used to estimate the linear equations between gene transcripts of two types of receptor and some biochemical markers. The logistic regression model was constructed to assess the effects of cardiac levels of AT$_R$ and AT$_2R$ gene transcripts: single quadratic term, linear plus quadratic term, logarithm, exponential, and square root. The simple linear term fit the data best as per Akaike’s Information Criterion.\(^{17}\) The estimates of these logistic models were used to calculate the receiver operating characteristic (ROC) curves. The area under the curve was estimated using the Hanley and McNeil method.\(^{18}\) The area under the curve is a measure of the discriminatory power of the equation and ranges from 0.5 to 1.0 with 1.0 as perfect and 0.5 no better than chance. Differences were considered significant at $P < 0.05$. Due to the small sample size of this exploratory study, no adjustment for other confounders was made and no model building strategy was adopted.
Results

Total study population

The mean age of recipients was 55.5 years (±12.8 SD). Five patients whose endomyocardial biopsies did not yield sufficient total RNA were excluded from the analysis. Thirty-two recipients had hypertension, including seven who presumably had cyclosporine-induced hypertension. There were no differences in systolic (normotensives: 131 ± 12; treated hypertensives: 141 ± 14; treated cyclosporine-induced hypertensives 145 ± 20 mm Hg; P = 0.33) or diastolic blood pressure (normotensives: 78 ± 10; treated hypertensives: 88 ± 11; treated cyclosporine-induced hypertensives 84 ± 12 mm Hg; P = 0.24) in the normotensive and treated hypertensive populations. Twenty-six patients were commenced on angiotensin I converting enzyme (ACE) inhibitors after transplantation, for the treatment of hypertension. The remaining patients with hypertension were on other antihypertensive medications, including diltiazem, amlodipine, and doxazocine. None of the patients was on Ang II receptor blocker. Eleven patients had diabetes, including five whose diabetes was presumably induced by steroids. There were no differences in blood glucose concentration between non-diabetics and diabetics (non-diabetics 92 ± 17 mg/dl; steroid-induced diabetics 116 ± 30 mg/dl; other diabetics 127 ± 62 mg/dl; P = 0.14). Mean total cholesterol was 183 ± 44 mg/dl and low density lipoprotein cholesterol was 98 ± 39 mg/dl. Forty-two patients were on statins for the treatment of hypercholesterolaemia. All patients received standard triple immunosuppressive drugs, including prednisone (dose: 7.2 ± 3.8 mg per day), cyclosporine (dose: 149 ± 67 mg; plasma level 215 ± 52 ng/ml), and mycophenolate mofetil (dose 1463 ± 688 mg; plasma level 2.7 ± 1.3 mg/l). Table 1 depicts the clinical and laboratory characteristics of the study population. Table 2 summarizes the endomyocardial biopsy and IVUS results in groups of patients defined by the presence or absence of vasculopathy one year after transplantation. In the overall study population the mean values were: average biopsy score 1.26 ± 0.51, CMIT 0.47 ± 0.37 mm, CPV 4.9 ± 2.8 mm³, AT1R 2.6 ± 2.5 folds, AT2R 0.69 ± 0.74 folds. Cardiac AT1R gene transcript level positively correlated with that of AT2R, as shown in Fig. 2. As shown in Table 3 and Fig. 3, linear regression analyses demonstrated associations between IVUS indices of TCAD, measured as CMIT or CPV, and cardiac AT1R or AT2R mRNA levels. There was no association between recipient age and CMIT (r = 0.05; P = 0.75) or CPV (r = 0.14; P = 0.33). Level of systolic or diastolic blood pressure, serum cholesterol (total or LDL fraction), and plasma level of cyclosporine were not associated with cardiac AT1R or AT2R gene transcripts, as shown in Table 3. However, the plasma level of mycophenolate mofetil was inversely related to level of AT2R gene transcripts.

Acute rejection

The entire study population developed one or more episodes of acute rejection during the first year after transplantation. At one year after transplantation, 20 patients developed grade IA, two patients grade IB, 4 patients grade II and four patients grade IIIA acute rejection. The remaining patients showed no evidence of acute rejection. Acute rejection was treated with pulsed steroids for three days. There was no difference in the level of AT1R (P = 0.70) and AT2R (P = 0.50) gene transcripts one year after transplantation in these groups. No correlation was observed between average biopsy score and CMIT (r = 0.04; P = 0.78) or CPV (r = 0.14; P = 0.32).

Transplant vasculopathy

(A) Based on predefined CMIT criteria, 21 patients (47%) developed vasculopathy, in other words, a CMIT of 0.3 mm or more at one-year follow-up. This vasculopathic group was compared with the remaining cohort of 24 recipients (53%), who did not develop vasculopathy at one year of follow-up. As shown in Tables 1 and 2, there were no differences in baseline patient characteristics, including age, number of patients treated with ACE

Table 1 Clinical and laboratory characteristics of the study population

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total study population (n = 45)</th>
<th>Patients with transplant vasculopathy (n = 21)</th>
<th>Patients without transplant vasculopathy (n = 24)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor age (years)</td>
<td>35 ± 14</td>
<td>40 ± 14</td>
<td>30 ± 12</td>
<td>0.01</td>
</tr>
<tr>
<td>Recipient age (years)</td>
<td>56 ± 13</td>
<td>56 ± 13</td>
<td>57 ± 10</td>
<td>0.90</td>
</tr>
<tr>
<td>Ischaemic time (min)</td>
<td>171 ± 53</td>
<td>165 ± 48</td>
<td>177 ± 58</td>
<td>0.50</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>138 ± 17</td>
<td>138 ± 16</td>
<td>139 ± 18</td>
<td>0.90</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>85 ± 11</td>
<td>83 ± 11</td>
<td>87 ± 12</td>
<td>0.40</td>
</tr>
<tr>
<td>Total cholesterol (mg)</td>
<td>183 ± 44</td>
<td>182 ± 48</td>
<td>184 ± 42</td>
<td>0.86</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>98 ± 39</td>
<td>103 ± 44</td>
<td>96 ± 36</td>
<td>0.63</td>
</tr>
<tr>
<td>Blood glucose (mg)</td>
<td>99 ± 30</td>
<td>97 ± 23</td>
<td>100 ± 35</td>
<td>0.72</td>
</tr>
<tr>
<td>Number of patients on statins</td>
<td>42</td>
<td>19</td>
<td>23</td>
<td>0.31</td>
</tr>
<tr>
<td>CMV</td>
<td>13</td>
<td>8</td>
<td>5</td>
<td>0.12</td>
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</table>

TCAD: transplant coronary artery disease; CMV: cytomegalovirus.
inhibitors, average biopsy score, and baseline intimal thickening between the vasculopathic and non-vasculopathic groups. The mRNA level of AT1R, but not AT2R, in endomyocardial biopsy samples was significantly higher in patients who demonstrated vasculopathy in comparison with those who showed no vasculopathy (3.7/2.9 vs 1.6/1.7 folds; P = 0.006). Regardless of treatment with or without ACE inhibitors, the levels of AT1R gene transcripts were elevated in the patients in the vasculopathic group compared to those with non-vasculopathy at one year.

(B) At the time of this study, 26 transplant recipients (58%) were on ACE inhibitors and the remaining 19 patients (42%) were on standard immunosuppressive drugs alone. Patients who were treated with ACE inhibitors and those who were not were comparable in age, average biopsy score, and baseline intimal thickening. There were no differences in cardiac AT1R and AT2R mRNA levels one year after transplantation.

Analysis of receiver operating characteristic curves

The estimated ROC curve values for AT1R gene transcript were: area under the ROC curve 0.69, sensitivity 75%, specificity 83%, positive predictive value 79%, and negative predictive value 79% (see Fig. 4). The area under the ROC curve for the AT1R gene transcript was 0.44 so the other calculations were not made. The effect of AT1R and AT2R gene transcripts on the odds of developing transplant vasculopathy are shown in Table 4. A unit change in the level of AT1R gene transcripts leads to odds of 36% of developing vasculopathy. On the other hand, AT2R mRNA is not significantly associated with the occurrence of transplant vasculopathy, although there is a tendency to vasculopathy when AT2R transcripts are elevated.

Discussion

In the present study, we observed that the levels of cardiac AT1R gene transcripts are directly related to
those of AT2Rs in transplanted hearts. Both AT1R and AT2R gene transcripts in transplanted heart predicted the progression of TCAD one year after transplantation. Recipients who had developed vasculopathy one year after transplantation demonstrated a higher level of AT1R gene transcripts in comparison with those who showed no vasculopathy. A unit change in the level of AT1R gene transcripts in the transplanted heart was associated with odds of 36% of developing transplant vasculopathy. These findings suggest that Ang II receptors might contribute to the progression of TCAD following heart transplantation.

Numerous clinical and laboratory data support the hypothesis that activation of tissue RAS is particularly important in mediating long-term effects on cardiovascular system.19,20 The interaction of AT1R and AT2Rs in

Table 3 Results of linear regression analysis

<table>
<thead>
<tr>
<th>Variables</th>
<th>Ang II type 1 receptor transcripts</th>
<th></th>
<th>Ang II type 2 receptor transcripts</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Regression coefficient</td>
<td>95% CI</td>
<td>P-value</td>
<td>Regression coefficient</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------------</td>
<td>---------</td>
<td>---------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Regressor</td>
<td>Lower</td>
<td>Upper</td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td>Ang II type 1 receptor transcripts</td>
<td>0.10</td>
<td>0.06</td>
<td>0.14</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CMIT</td>
<td>0.41</td>
<td>0.17</td>
<td>0.65</td>
<td>0.001</td>
</tr>
<tr>
<td>CPV</td>
<td>0.31</td>
<td>−5.45</td>
<td>6.07</td>
<td>0.913</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>1.90</td>
<td>−3.10</td>
<td>6.90</td>
<td>0.444</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>4.56</td>
<td>−3.95</td>
<td>13.06</td>
<td>0.284</td>
</tr>
<tr>
<td>Plasma level of cyclosporine</td>
<td>−0.19</td>
<td>−0.50</td>
<td>0.11</td>
<td>0.209</td>
</tr>
<tr>
<td>Plasma level of mofetil</td>
<td>0.37</td>
<td>−0.15</td>
<td>0.88</td>
<td>0.156</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>0.19</td>
<td>0.04</td>
<td>0.01</td>
<td>0.029</td>
</tr>
</tbody>
</table>

CI: confidence interval, regression coefficient represents the direction and magnitude of the effects of Ang II type 1 and type 2 receptor transcripts on outcome variables; Ang II: angiotensin II; CMIT: change in maximal intimal thickness; CPV: change in plaque volume; LDL: low-density lipoprotein; AT1R: angiotensin II type 1 receptor.

Fig. 3 Scatter plots showing relationship between intravascular ultrasound (IVUS) indices of transplant coronary artery disease and mRNA expressions of angiotensin II type 1 (AT1R) and type 2 (AT2R) receptors: upper panel shows the relationship between change in maximal intimal thickness and AT1R and AT2R and lower panel shows the relationship between change in plaque volume and AT1R and AT2R. IVUS measurements were obtained at the worst affected sites in the course of coronary arteries.
mediating the biological effects of Ang II is not well understood. Increased AT1R activity may down-regulate the expression of AT2R, possibly through increased production of growth factors such as platelet-derived growth factor and epidermal growth factor. Conversely, inhibition of AT1R causes an activation of AT2R and may thus contribute to at least some of the effects of AT1R antagonists. Although, the vast majority of Ang II functions in the cardiovascular system are attributable to AT1R, a number of recent reports suggest a possible role of AT2R under physiological or pathological states. For instance, AT2R may be essential for the development of left ventricular (LV) hypertrophy and fibrosis in chronic Ang II-induced hypertension. Both AT1R and AT2R may contribute to LV hypertrophy independent of loading conditions.

AT1R and AT2Rs are expressed in normal human heart and are important in its physiology and the progression to myocardial hypertrophy. Autoradiographic studies have demonstrated that AT2R is the dominant receptor in the normal human heart. Heart failure is associated with selective down-regulation of AT1R in proportion to the degree of ventricular dysfunction, producing either no change or up-regulation of AT2R. These alterations in gene expression in heart failure lead to an increase in the distribution ratio between AT1Rs and AT2Rs. Receptor density may define the biological efficacy of Ang II, with an increase in receptor expression associated with enhanced biological effects of Ang II.

The present study was not designed to determine the differences in the levels of Ang II receptor gene transcripts between normal and transplanted hearts. However, using normal heart RNA as a reference, we found (1) increased levels of cardiac AT1R gene transcripts and (2) an association between AT1R and AT2R gene transcripts in transplanted hearts. A similar pattern of AT1R and AT2R gene transcripts was observed in native left ventricular hypertrophy. The data of the present study indicate that both AT1R and AT2R are possibly involved in the progression of TCAD. Several observations support the role of Ang II receptors in the pathogenesis of vasculopathy. For example, a number of experimental and clinical studies indicated that both AT1R and AT2R are involved in the pathogenesis of conventional atherosclerosis in the coronary arteries. AT2R expression, which is dramatically decreased after birth, reappears in vascular lesions, suggesting a role of these receptors in the regulation of atherogenesis and vascular remodeling. AT1R occupancy stimulates multiple intracellular signalling cascades that result in smooth muscle cell hypertrophy/hyperplasia, synthesis of extracellular matrix, production of inflammation, and immune modulation, and may thus contribute to the progression of intimal thickening. On the other hand, activation of AT2R triggers an anti-growth effect and modulates AT1R-induced vascular smooth muscle cell proliferation and extracellular matrix accumulation. There is some evidence that AT2R stimulation may increase collagen synthesis in vascular smooth muscle cells. These observations may suggest a potentially interacting role of AT1Rs and AT2Rs in the pathogenesis of conventional atherosclerosis.

We propose a similar role for AT1R and AT2R in the initiation and progression of TCAD. Consistent with this concept, experiments using animal transplant models demonstrated that AT1R blockade significantly decreases intimal proliferation of coronary arteries after heart transplantation. ACE gene polymorphism in donor or recipient correlate with additional risk for the development of TCAD after heart transplantation.

The role of immune mechanisms in mediating TCAD progression is not completely understood. Evidence suggests that the incidence of TCAD has, indeed, increased following the introduction of cyclosporine. Moreover, reports investigating the association between allograft rejection and TCAD produced conflicting results. The findings in the present study showed no association between recipient’s immune response, measured as the average biopsy score over one year, or acute rejection episodes and the progression of TCAD. The interaction between RAS and immune mechanisms has been investigated in a series of recent studies. RAS may both stimulate and be stimulated by alloimmune responses. Immune cells synthesize Ang II and express Ang II receptors. Ang II may function as an autocrine factor for promoting T-cell proliferation. Furthermore, treatment with immunosuppressives, including cyclosporine A, suppresses Ang II-induced organ damage and AT1R antagonists reduce the risk of chronic rejection in animal transplant models. Using a competitive RT-PCR method, an earlier report showed down-regulation of both AT1R and AT2Rs after heart transplantation. The discrepancy between these findings and those of our data may be attributable to differences in study conditions.

Table 4 The effect of AT1R and AT2R transcript levels on the odds of developing transplant vasculopathy

<table>
<thead>
<tr>
<th>Variables</th>
<th>Odds ratio</th>
<th>95% Confidence interval</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1R</td>
<td>1.36</td>
<td>1.00–1.85</td>
<td>0.049</td>
</tr>
<tr>
<td>AT2R</td>
<td>1.16</td>
<td>0.49–2.76</td>
<td>0.730</td>
</tr>
</tbody>
</table>

Fig. 4 Receiver operating characteristic (ROC) curves for cardiac AT1R and AT2R levels in predicting the development of transplant vasculopathy one year after transplantation.
design, including the size of the study population, timing of endomyocardial biopsy, and analytic methods used for gene expression analysis.

Using a CMIT of $\geq 0.3 \text{mm}$ over one year as a criterion to classify the transplant vasculopathy, cardiac AT$_1$R, but not AT$_2$R, gene transcription was identified as a discriminator in predicting vasculopathy with high sensitivity (75%) and specificity (83%) following transplantation.

Cardiac AT$_1$R or AT$_2$R gene transcription in the present study did not differ between recipients treated and untreated with ACE inhibitors in agreement with some previous studies. These findings suggest that incomplete blockade of the conversion of Ang I to Ang II or activation of alternative pathways such as chymases for the generation of Ang II might be important in heart transplant recipients who were treated with ACE inhibitors for hypertension. Several studies suggest a gradual reactivation of ACE activity over time with the use of ACE inhibitors. Consistent with this observation and the subsequent increase in the sensitivity of Ang II receptors, the addition of an AT$_1$R blockade to a chronic ACE inhibitor treatment may produce a clinical and haemodynamic benefit in heart failure patients. These observations support our findings that AT$_1$R gene transcriptions significantly and positively correlated with the indices of TCAD regardless of treatment with ACE inhibitors.

Our earlier study demonstrated an association between TCAD progression and levels of cardiac vitronectin receptors. Vitronectin receptor is activated by Ang II through AT$_1$R. In the present study we extend these observations to AT$_1$R and AT$_2$R gene transcripts. Based on our observations and on earlier published reports, we support a RAS hypothesis for the development and progression of TCAD after heart transplantation.

**Study limitations**

This was an observational study and our conclusions are based on correlation and regression analysis, which do not establish a causal relationship between Ang II receptors and the development of TCAD. The amount of surveillance endomyocardial biopsy specimens precludes assessment of AT$_1$R or AT$_2$R at protein level and several other components of RAS.

**Conclusions**

The findings of the present study highlight the importance of cardiac Ang II receptors in the progression of intimal thickening and the development of transplant vasculopathy in recipients of heart transplantation. Both AT$_1$R and AT$_2$R gene transcripts in transplanted heart predict the progression of TCAD in heart transplant recipients. Elevated levels of AT$_1$R gene transcripts in transplanted heart confer an increased risk of transplant vasculopathy in recipients of heart transplantation. These data provide evidence for a potential role of cardiac Ang II receptor gene transcripts, particularly AT$_1$R transcripts, in the development of transplant vasculopathy in recipients of heart transplantation. The present evidence potentially extends the renin-angiotensin concept of conventional atherosclerosis to transplant vasculopathy and supports a role of non-immunological factors in the pathogenesis of the latter. However, further studies are needed to elucidate possible aberrations in AT$_1$R- or AT$_2$R-mediated signal transduction pathways and their interaction with the immune system in patients with TCAD after transplantation to better understand their mechanisms and to develop precisely targeted treatment.

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**References**

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