Gene Sequencing and Tissue Expression of Unknown Isoforms of an Angiotensin II Type 2 Receptor Interacting Protein, ATIP, in the Rat

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To investigate the expression of the unknown angiotensin II type 2 receptor interacting protein (ATIP) isoforms in the rat we used the known sequences of human and mouse ATIP to design sequencing primers to enable us to sequence rat ATIP3 and ATIP4. Exon 4, which is present in human but not mouse ATIP, was not identified in the coding region of rat ATIP. The expression levels of these genes in a range of rat tissues were examined, and we concluded that there is little similarity in the relative tissue distribution of the various ATIP isoforms in rat and human.

Key words: angiotensin II type 2 receptor interacting protein; gene expression; quantitative PCR; rat; sequencing

Loss of heterozygosity at chromosome 8p21.3-22 is common in a range of tumors, including cancers of the prostate, bladder, breast, ovary, colon, liver, and head and neck.1–5) Recently, using large-scale genomic DNA sequencing6) and differential display reverse transcription PCR,7) a candidate tumor suppressor gene was identified in this region. It encodes a family of five proteins collectively known as the angiotensin II (Ang II) type 2 receptor (AT2-receptor) interacting protein (ATIP).8–10) The five isoforms are numbered 1, 2, 3a, 3b and 4,10) and are alternatively known as the mitochondrial tumour suppressor gene (MTUS1/MTSG1),7) the AT2-receptor binding protein of 50 kDa (ATBP50),11) and GK1.6)

The human ATIP gene spans 112 kb of genomic DNA, across 17 exons. The largest exons, 1 and 17, are 2,245 bp and 2,345 bp respectively, while the remaining 15 exons range from 39 bp (exon 7) to 510 bp (exon 5). Alternative exon usage generates five transcript variants that are grouped as three major species, ATIP1, ATIP3 (2, 3a and 3b) and ATIP4.8,9) The structural organization of these exons can be seen in Fig. 1 of Di Benedetto et al.8) Exon 1 is a 5′ coding exon containing a 5′ untranslated region and a translational ATG codon for ATIP2, 3a and 3b, while exons 5 and 8 are 5′ coding exons for ATIP4 and ATIP1 respectively. The locations of these initiating codons suggest that the variants have different promoters, as confirmed by the varied tissue distributions of ATIP1, ATIP3, and ATIP4.8,9)

In humans, the highest levels of ATIP mRNA are found in the brain and placenta. There are significant levels of ATIP in all other tissues, except for the bone marrow which contains little or no ATIP expression. On average, combined ATIP3a and ATIP3b expression accounts for 72% of total ATIP expression in most tissues, and these are the major transcripts in all human tissues except the brain. ATIP3a accounts for 47% of total ATIP expression, whereas ATIP3b accounts for 25%. By contrast, ATIP1 expression levels vary widely, ranging from 3 to 70%, and totalling 28% of all ATIP expression. ATIP4 mRNA is present only in the brain, accounting for 0.15% of total ATIP expression, whereas ATIP2 expression is less than 1% in all tissues studied to date.8)

Studies indicate that ATIP interacts with the C-terminal tail of the AT2-receptor,10,11) and identify ATIP as a possible intracellular messenger for this receptor as well as a novel inhibitor of growth-factor signalling.9) For instance, overexpression of ATIP, in eukaryotic cells that contain AT2-receptors, inhibits activation of extracellular signal-regulated kinase 2, mimicking the activation of the AT2-receptor in these cells.10,11)

In addition, we found recently that ATIP is involved in tumor suppression through inhibition of growth factors such as endothelial growth factor in prostate cancer cell lines.12) In regard to the specific functions of the various isoforms, it has been found that both ATIP112) and ATIP313) possess tumor suppressor-like actions, indicating that this is a characteristic of all ATIP variants.

Excessive growth activity is not confined to cancer cells, however, but contributes to morbidity in a wide range of diseases, including diabetes and cardiac disease. We wish to study these diseases, and in particular the role of ATIP in the progression of these conditions, in rat models of hypertension, cardiac disease, and diabetes.14–18) However, although all variants of human and mouse ATIP have been sequenced, other than rat ATIP1, little is known concerning ATIP.
and the relative expression of its isoforms in the rat.
Here we report the sequencing of the unknown isoforms of rat ATIP, detail their relative expression in a wide range of rat tissues, and compare their localization with what has previously been described for humans.8

**Materials and Methods**

Cell culture. RNA extraction, DNase treatment, and cDNA synthesis in a rat cell line. The mRNA of a rat cell line, normal rat kidney epithelial cells (NRK-52E),19 was used to generate a cDNA template for PCRs. Briefly, NRK-52E cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Mulgrave, Australia), supplemented with 4 mM t-glutamine and 10% fetal bovine serum, and kept at 37°C in a humidified atmosphere of 5% CO2/95% air. NRK-52E cells were grown to confluence in 75-cm2 culture flasks, cells were harvested, and mRNA extraction, DNase treatment, and cDNA synthesis were performed as previously described.20

**PCR primer design for rat ATIP3 and rat ATIP4.** The template used for designing rat ATIP3 PCR and sequencing reaction primers was an obsolescent version of the rat ATIP sequence obtained from GenBank (GenBank accession no. NM_573915). We determined that this sequence (which has recently been replaced with the sequence for rat ATIP1, GenBank accession no. NM_178093), shared 72.3% coding region homology with the sequence of human ATIP3b (GenBank accession no. NM_001001925), indicating that it is a suitable template from which to design PCR and sequencing reaction primers. The sequences for human ATIP4 (GenBank accession no. NM_001001931) and mouse ATIP4 (GenBank accession no. NM_0010055864) were aligned to identify regions of shared sequence that were also unique to these respective ATIP4 variants. While these parameters proved restrictive, a suitable template was ultimately identified from which rat ATIP4 PCR and sequencing reaction primers were designed. All primers were purchased from Sigma-Aldrich (St. Louis, MO).

**PCR and sequencing reaction for rat ATIP3.** Using GoTaq® Mastermix (Promega, Madison, WI), regions of rat ATIP3 were amplified, and the resulting PCR products were run on a 1% agarose gel (Sigma-Aldrich) at 70 V for 90 min. Separated bands were excised from the gel using a DNA extraction kit (Qagen Sciences, German-town, MD), and the purified product was sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA).

**PCR, cloning reaction, and sequencing reaction for rat ATIP4.** PCR of rat ATIP4 was performed as described for rat ATIP3, but the PCR product yield was insufficient to perform successful sequencing reactions. Consequently, purified product and PCR control product were ligated into the TOPO® TA cloning vector (Invitrogen) and transformed into One Shot® competent cells (Invitrogen), as detailed in the supplier’s instructions. The transformants were incubated overnight at 37°C on an agar plate selective for kanamycin, and six random ATIP4 transformants were chosen for analysis. Plasmid DNA was isolated using a DNA extraction kit (Qagen), and restriction digest was performed to identify insertion of the PCR product. Constructs containing the purified product were sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). The rat ATIP3 and rat ATIP4 extension products were purified and then sequenced by the University of Melbourne, Department of Pathology Sequencing Service (Melbourne, Australia).

**RNA extraction, DNase treatment, cDNA synthesis, and quantitative PCR (QPCR) for rat tissue.** Following the sequencing of rat ATIP3 and rat ATIP4, Taqman® QPCR probes and primers (Table 1) were designed using Primer Express v2.0 (Applied Biosystems) to make possible quantitative examination of rat ATIP, ATIP1, ATIP3, and ATIP4 mRNA expression in a range of rat tissues (Table 2). Briefly, 16-week-old male Sprague-Dawley (SD) rats, each weighing approximately 350 g, were sacrificed using halothane, a range of tissues were removed, and mRNA extraction, DNase treatment, and cDNA synthesis were performed as previously described.20 QPCRs for all the cDNA samples (for each tissue type, n = 6) were then conducted using an Applied Biosystems 7500 Real-time PCR system. To adjust for any variation in the amount of cDNA added per PCR, target gene expression was normalized by quantifying the mRNA expression of a housekeeping gene, 18S RNA (18S rRNA TaqMan Control Reagent, Applied Biosystems). PCR amplification was performed as previously described.20

ΔCt values were determined as previously described.20 Briefly, the Ct value for the control housekeeping gene, 18S, was subtracted from the Ct value for the target gene (ATIP, ATIP1, ATIP3, or ATIP4), producing a ΔCt value; the lower this value, the higher the level of mRNA expression. Mean ΔCt values and SE values were calculated for all tissues.

**Rat tissue lysate preparation and western blotting for ATIP protein determination.** Several of the excised SD rat tissues were selected for ATIP protein expression determination. Selection was based primarily on the importance of the tissue for survival as well as ease of lysate preparation. Briefly, tissues were homogenized in an ice-cold buffer consisting of 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 1 mM EGTA, 25 mM sucrose, 5% glycerol, 50 mM β-mercaptoethanol, 2 mM PMSF, and 0.1% nonidet P-40. The protein concentration of the lysates was determined by Bradford protein assay21 before the 1:1 addition of a modified Laemmli’s final sample buffer:22 50 mM DTT, 20% glycerol, 125 mM Tris–HCl (pH 6.8), 4% SDS, and 0.01% bromophenol blue. Protein denaturation was completed by heating the samples for 5 min at 95°C. Lysates (for each tissue type, n = 3) were separated by SDS–PAGE on precast gels supplied by Bio-Rad Laboratories (Hercules, CA) containing 10% acrylamide, and transferred to a nitrocellulose membrane. The membrane was blocked with PBS containing 2% skim-milk/2% Tween 20 and incubated overnight at 4°C with 4 μg/mL of anti-ATIP antibody (Abnova, Taipei, Taiwan). ATIP protein bands were visualized by chemiluminescence (ECL)+ Western blotting detection reagents, GE Healthcare, Chalfont St. Giles, UK; LAS-3000 imaging system, Fujifilm, Tokyo), and then the membranes were stripped of the target protein antibody and rebiurized with a control antibody (anti-GAPDH, Sigma-Aldrich), to correct for the amount of protein added. Protein band intensities were analyzed with Science Lab 2003 MultiGauge software (Fujifilm), and the ratio of ATIP over GAPDH was calculated.

### Results and Discussion

#### Sequencing of rat ATIP3 and rat ATIP4

Using the obsolete rat ATIP sequence as a template for rat ATIP3 and the human and mouse ATIP4 sequences as a template for rat ATIP4, we were able to identify the complete coding sequences of the two genes (GenBank accession nos. EU417559 for rat ATIP3, and GU245886 for rat ATIP4). We were able to identify only a single ATIP3 variant in the rat, which corresponds to ATIP3b in human. The sequence of exon

**Table 1. Sequences of Probes and Primers Used in Quantitative PCR (QPCR)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Probe</th>
<th>Fwd</th>
<th>Rev</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat ATIP Fwd</td>
<td>CTGGTTGGAACACAC</td>
<td>GTGACGGTGAAGATGG</td>
<td></td>
</tr>
<tr>
<td>Rat ATIP Rev</td>
<td>GAGGTGGAGATGTTA</td>
<td>CGTGATGGCGACGTGA</td>
<td></td>
</tr>
<tr>
<td>Rat ATIP3 Fwd</td>
<td>GCACCAAGCACTGAT</td>
<td>CGTCAGGGCGTACCT</td>
<td></td>
</tr>
<tr>
<td>Rat ATIP3 Rev</td>
<td>GCCACAGCTACTGAC</td>
<td>CCGCCGTCGCTCA</td>
<td></td>
</tr>
<tr>
<td>Rat ATIP4 Fwd</td>
<td>GACGATCTGTTATCAATTCTACCTCAA</td>
<td>CCGAGCTGATCAATTTGAAGTCAGCA</td>
<td></td>
</tr>
</tbody>
</table>

Fwd, forward primer; Rev, reverse primer.
4, which is present in human ATIP3a but not ATIP3b, is not present in the rat coding region for ATIP3, indicating that this gene and the protein it encodes are not present in the rat. The absence of exon 4 from the ATIP coding region is also observed in the mouse, which was expected, as mouse and rat ATIP3 share 89.7% coding sequence homology and 90.5% amino acid sequence homology (allowing for conservative amino acid substitution). A schematic representation of the exon composition of these isoforms, and how they correspond to the human isoforms, is shown in Fig. 1.

Alignment of human ATIP3b and ATIP4 with rat ATIP3 and ATIP4

The nucleotide and amino acid sequences of rat ATIP3 and rat ATIP4 were aligned with their corresponding sequences in humans to determine the homology shared by the species (Supplemental Figs. 1, 2, 3, and 4; see Biosci. Biotechnol. Biochem. Web site). Using the alignment function at the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) website,23 we determined that rat ATIP3 and human ATIP3b share 75.1% nucleotide homology within their coding regions, from start to stop codon, and 75.7% amino acid sequence homology (allowing for conservative amino acid substitution), while rat ATIP4 and human ATIP4 share 84.0% nucleotide homology within their coding regions, and 90.8% amino acid sequence homology (allowing for conservative amino acid substitution).

ATIP, ATIP1, ATIP3, and ATIP4 mRNA expression in rat tissue

In the SD rat, individual gene expression varied significantly between the various tissues, with mean ΔCT values for total ATIP expression ranging from 11.1 ± 0.6 in the heart to 17.0 ± 0.3 in the duodenum. Total ATIP expression was also high in the lung and skeletal muscle, with ΔCT values of 11.5 ± 0.3 and 11.9 ± 0.1 respectively (Table 2). There was also significant variation between ATIP1, ATIP3, and ATIP4 mRNA expression (Table 2) in most tissues, the mean ΔCT values for the heart being 13.9 ± 0.5, 16.6 ± 0.5, and 22.0 ± 0.6 respectively.

We analyzed the ΔCT values for total ATIP to determine the ATIP values relative to the tissue with the lowest ATIP mRNA expression, the duodenum (Table 3). Using these values in conjunction with the ΔCT values for ATIP1, ATIP3, and ATIP4, we established an expression profile of the variants across a range of tissues (Table 3 and Fig. 2). Since ATIP2 expression is less than 1% in all the human tissues so far studied,86 possibly because the coding region contains an

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**Table 2.** ATIP, ATIP1, ATIP3, and ATIP4 mRNA Expression in 16-Week-Old Male Sprague-Dawley (SD) Rat Tissues

<table>
<thead>
<tr>
<th>SD rat tissue</th>
<th>ATIP</th>
<th>ATIP1</th>
<th>ATIP3</th>
<th>ATIP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>11.1</td>
<td>13.9</td>
<td>16.6</td>
<td>22.0</td>
</tr>
<tr>
<td>Lung</td>
<td>11.5</td>
<td>13.8</td>
<td>14.9</td>
<td>19.6</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>11.9</td>
<td>16.4</td>
<td>13.2</td>
<td>19.5</td>
</tr>
<tr>
<td>Sk Muscle</td>
<td>11.9</td>
<td>15.5</td>
<td>15.5</td>
<td>19.6</td>
</tr>
<tr>
<td>Adrenal</td>
<td>12.0</td>
<td>16.2</td>
<td>14.7</td>
<td>20.3</td>
</tr>
<tr>
<td>Spinal Cord</td>
<td>12.7</td>
<td>14.9</td>
<td>16.7</td>
<td>20.0</td>
</tr>
<tr>
<td>Liver</td>
<td>13.0</td>
<td>17.8</td>
<td>15.8</td>
<td>20.5</td>
</tr>
<tr>
<td>Bladder</td>
<td>13.2</td>
<td>17.3</td>
<td>15.5</td>
<td>19.7</td>
</tr>
<tr>
<td>Testes</td>
<td>13.4</td>
<td>17.3</td>
<td>16.6</td>
<td>19.0</td>
</tr>
<tr>
<td>Brain</td>
<td>13.6</td>
<td>15.8</td>
<td>18.1</td>
<td>19.9</td>
</tr>
<tr>
<td>Stomach</td>
<td>14.1</td>
<td>19.4</td>
<td>16.1</td>
<td>21.6</td>
</tr>
<tr>
<td>Kidney</td>
<td>14.4</td>
<td>18.1</td>
<td>16.2</td>
<td>21.8</td>
</tr>
<tr>
<td>Spleen</td>
<td>16.5</td>
<td>18.8</td>
<td>21.2</td>
<td>24.5</td>
</tr>
<tr>
<td>Colon</td>
<td>16.9</td>
<td>20.8</td>
<td>19.0</td>
<td>24.3</td>
</tr>
<tr>
<td>Duodenum</td>
<td>17.0</td>
<td>21.0</td>
<td>19.3</td>
<td>24.6</td>
</tr>
</tbody>
</table>

All values are presented as mean ΔCT ± SE. The primers and probe for ATIP bound to a homologous region within the three known rat ATIP variants, and hence the values represent a measure of total ATIP mRNA present.

**Table 3.** Relative Expression of ATIP Isoforms in 16-Week-Old Male Sprague-Dawley (SD) Rat Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>ATIP value</th>
<th>ATIP1 %</th>
<th>ATIP3 %</th>
<th>ATIP4 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>58</td>
<td>86</td>
<td>14</td>
<td>0.3</td>
</tr>
<tr>
<td>Lung</td>
<td>44</td>
<td>68</td>
<td>31</td>
<td>1</td>
</tr>
<tr>
<td>Oesophagus</td>
<td>35</td>
<td>9</td>
<td>90</td>
<td>1</td>
</tr>
<tr>
<td>Sk Muscle</td>
<td>34</td>
<td>48</td>
<td>48</td>
<td>3</td>
</tr>
<tr>
<td>Adrenal</td>
<td>31</td>
<td>26</td>
<td>72</td>
<td>1</td>
</tr>
<tr>
<td>Spinal Cord</td>
<td>20</td>
<td>76</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td>Liver</td>
<td>16</td>
<td>19</td>
<td>78</td>
<td>3</td>
</tr>
<tr>
<td>Bladder</td>
<td>14</td>
<td>21</td>
<td>75</td>
<td>4</td>
</tr>
<tr>
<td>Testes</td>
<td>12</td>
<td>33</td>
<td>56</td>
<td>11</td>
</tr>
<tr>
<td>Brain</td>
<td>11</td>
<td>80</td>
<td>16</td>
<td>5</td>
</tr>
<tr>
<td>Stomach</td>
<td>7</td>
<td>9</td>
<td>89</td>
<td>2</td>
</tr>
<tr>
<td>Kidney</td>
<td>6</td>
<td>20</td>
<td>78</td>
<td>2</td>
</tr>
<tr>
<td>Spleen</td>
<td>1</td>
<td>82</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Colon</td>
<td>1</td>
<td>22</td>
<td>74</td>
<td>4</td>
</tr>
<tr>
<td>Duodenum</td>
<td>1</td>
<td>24</td>
<td>74</td>
<td>2</td>
</tr>
</tbody>
</table>

ATIP value indicates normalized expression of ATIP mRNA relative to the ΔCT of the lowest expressing tissue (the duodenum).


The number of bps in the coding regions of rat ATIP1, ATIP3, and ATIP4, the number of amino acids these genes encode, and the corresponding sizes of the proteins prior to any post-translational modifications (PTM) can be seen in Table 4. This information, in conjunction with Western blot studies conducted in rat cell lines overexpressing the human ATIP1 gene (data not shown), indicates that the size of the ATIP1 protein is approximately 60 kDa, while the size of the ATIP3 protein has been previously identified as approximately 170 kDa. Currently, it is unknown whether the Abnova anti-ATIP antibody identifies a band corresponding to ATIP4, and hence the protein expression of rat ATIP4 has not been quantified.

Western blotting was used to determine the expression levels of the ATIP1 and ATIP3 proteins in a range of SD rat tissues (Fig. 3 and Table 4). As seen for ATIP mRNA expression, there was great variation in protein levels across the tissues, the highest in the testes and lowest in the brain. However, the relative amounts of ATIP1 and ATIP3 protein did not correlate with the relative amounts of the corresponding mRNA, all of the tissues examined exhibiting higher ATIP1 protein expression than ATIP3. The mRNA versus protein correlation coefficient values for ATIP1 and ATIP3 were 0.0163 and 0.0006, respectively, indicating a lack of relationship between gene translation and protein transcription for both isoforms. This finding is consistent with those of Tamuguchi et al., who concluded that a cell’s mRNA and protein copy number for any given gene are uncorrelated. Their study was conducted on prokaryotic bacterial populations, while we have investigated mammalian tissue, but the concept that the progression from nucleic acids to proteins is a stochastic process is not new, and endeavors to determine a quantifiable linkage between the two continue.

The sequencing of rat ATIP isoforms and the subsequent determination of their tissue distribution in...
a range of tissues presented here should make possible further research into the function of ATIP in commonly used rat models of various disease states. Future findings using these rat models concerning the possible preventative role ATIP plays in conditions such as diabetes and hypertension should be applicable in similar research on human cells and tissues, as we have found that a high degree of mRNA and protein sequence identity exists between the species across the ATIP isoforms.

Acknowledgments

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