

Genome-wide gene expression profiling identifies overlap with malignant adrenocortical tumours and novel mechanisms of inefficient steroidogenesis in familial ACTH-independent macronodular adrenal hyperplasia

Dear Editor

ACTH-independent macronodular adrenal hyperplasia (AIMAH) is a rare cause of late-onset sporadic Cushing's syndrome (CS; Lacroix 2009). Discordance between massive adrenal hyperplasia and relatively mild hypercortisolism is distinctive of AIMAH, ascribed to differential localisation of steroidogenic enzymes in clear and compact cells, resulting in inefficient steroidogenesis (Lacroix 2009). Thus, CS is the result of hypercortisolism due to a colossal increase in adrenocortical cell mass. Iodocholesterol scintigraphy typically shows bilateral uptake consistent with bilateral adrenal hyperfunction, although unilateral or asymmetrical uptake may occur, guiding adrenal selection for resection (Gagliardi *et al.* 2009). We and others have reported AIMAH kindreds providing compelling evidence for an inherited basis in some cases (Gagliardi *et al.* 2009). The genetic basis of familial AIMAH is unknown, although segregation analysis suggests autosomal dominant inheritance (Gagliardi *et al.* 2009).

Cortisol secretion in AIMAH tumours may be regulated by aberrantly expressed (non-ACTH) G-protein-coupled receptors (GPCR; e.g. vasopressin (VP); Lacroix 2009). Whilst the biological basis for aberrant GPCR expression is unknown, in a murine model, it sufficed for the development of a phenotype reminiscent of human AIMAH (Mazzucco *et al.* 2006). This suggests that aberrant GPCR expression occurs early if it is not primary in the pathogenesis of AIMAH; alternatively, it could represent an epiphenomenon of tumorigenesis.

The pathogenesis of AIMAH, a cytologically benign disease without propensity for metastasis, is unknown. Molecular studies of sporadic AIMAH suggest altered WNT and protein kinase A signalling and microRNAs in its pathogenesis (Bimpaki *et al.* 2010, Almeida *et al.* 2011).

To our knowledge, transcriptome profiling of familial AIMAH has not been performed.

We have previously reported an AIMAH kindred (AIMAH-01), the proband of which, aged 69 years, presented with florid CS (Gagliardi *et al.* 2009). Bilateral adrenalectomy (combined weight 130 g) was curative, but he died from recurrent sepsis. His siblings, aged 61 and 65 years, presented with early CS; whilst lacking a Cushingoid phenotype, they had hypertension, mood change and central obesity. Iodocholesterol scintigraphy guided adrenalectomy of the dominant gland (weights 55 and 50 g respectively). One sibling became eucortisolaemic and the other transiently hypocortisolaemic – hypertension improved in both. Limited aberrant receptor testing revealed that all had an aberrant cortisol response to VP (Gagliardi *et al.* 2009). RT quantitative PCR (RT-qPCR) demonstrated that the tumours overexpressed the VP receptor 1A (*AVPR1A*) and ectopically expressed *AVPR1B* (Gagliardi *et al.* 2009).

Using Affymetrix Human GeneChip HumanGene 1.0 ST arrays, we compared the transcriptomes of the three available familial AIMAH-01 tumours with two normal adrenal glands and analysed the data for differential expression and using Ingenuity Pathway Analysis, Gene Set Enrichment Analysis (GSEA) and Motif Activity Response Analysis (MARA). We performed RT-qPCR validation of selected differentially expressed genes (DEG). Our aim was to determine the molecular mechanisms involved in the pathogenesis of familial AIMAH and the inefficient steroidogenesis which epitomises the tumours.

There were 367 significant DEG (162 upregulated). Global gene expression differences between AIMAH-01 and the normal samples were greater than within-group differences in AIMAH-01 (Fig. 1). The 50 most up- and downregulated significant DEG are shown in the

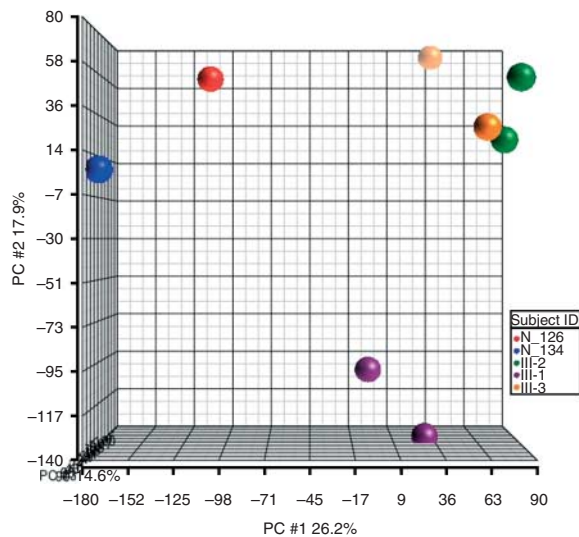


Figure 1 Principal component analysis plot of AIMAH-01 (III-1, III-2 and III-3) tumours and normal adrenal cortex (N_126 and N_134). The first principal component (PC #1) is shown on the x-axis and explains the maximum amount (26.2%) of variation in the gene expression data. The second principal component (PC #2) is shown on the y-axis and explains the second most amount of remaining variation in the gene expression data (17.9%). III-1, proband; III-2 and III-3, siblings. This figure was generated using Partek Genomics Suite, version 6.4 (Partek Incorporated, St Louis, MO, USA).

heat map (Fig. 2); we validated, by RT-qPCR, seven of the ten most DEG. The ACTH receptor was not differentially expressed. The glucocorticoid receptor was downregulated (2.5-fold; $P=0.056$).

Microarray analysis suggested reduced expression of the cholesterol side-chain cleavage enzyme (*CYP11A1*). Whilst not statistically significant by RT-qPCR (sevenfold, $P=0.09$), the downregulation may be biologically important in contributing to inefficient steroidogenesis because *CYP11A1* regulates the rate-limiting conversion of cholesterol to pregnenolone (Miller 2008). Furthermore, AIMAH is clinically and genetically heterogeneous; hence, more severe synthetic defects may be present in other cells (Lacroix 2009). Aldosterone synthase was downregulated (13-fold; $P=0.004$). The other steroidogenic enzymes studied were not differentially expressed.

Transcription factor activity profiling (MARA) predicted reduced activities of Sp1 transcription factor (*SPI*) and steroidogenic factor-1 (*NR5A1*), the transcriptional regulators of the rate-limiting enzymes of steroidogenesis, *CYP11A1* and steroidogenic acute regulatory protein (Miller 2008). However, *SPI* and *NR5A1* were not DEG. We postulate that reduced activity may be due to altered post-translational

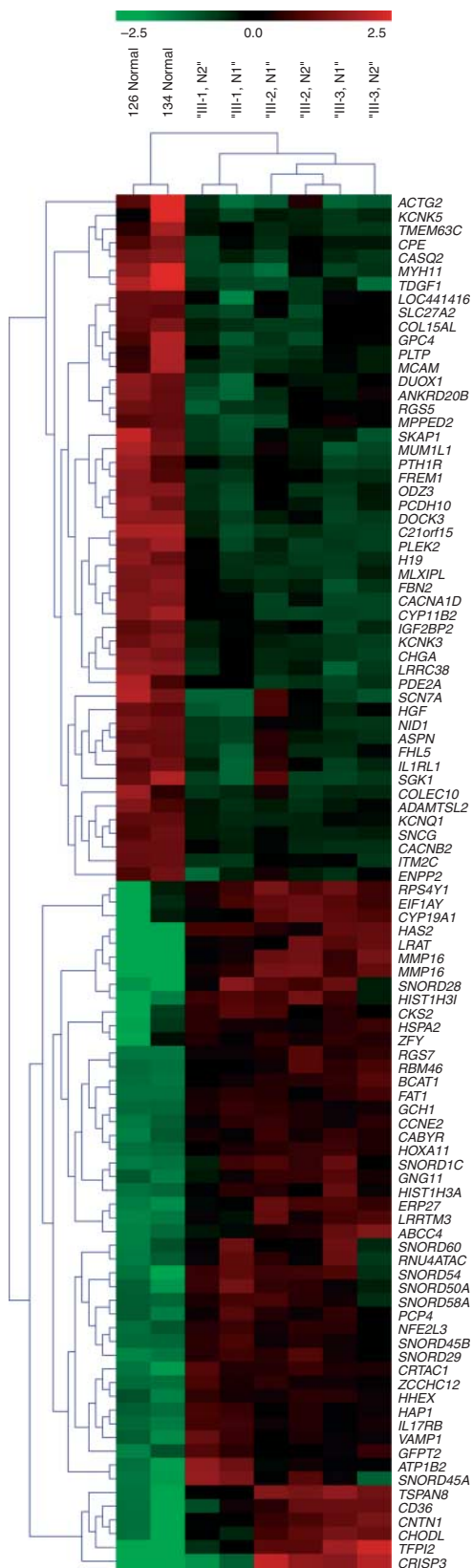
modification of their proteins (e.g. lowering activity, increasing degradation or altering interactions with coactivators). Downregulation of steroidogenic enzymes due to reduced transcription factor activity is a novel mechanism of inefficient steroidogenesis in AIMAH.

Most highly over-represented amongst the DEG was the ‘Cellular effects of Sildenafil’ signalling pathway. Sildenafil’s cellular target, *PDE5A*, was not a DEG. The microarray data suggested downregulation of *PDE1C* and *PDE2A*; these were not statistically significant by RT-qPCR. Nevertheless, the downregulation could be biologically significant, increasing intracellular cAMP signalling. These data suggest that dysregulated cAMP signalling, already implicated in adrenocortical tumorigenesis, may also be involved in the pathogenesis of AIMAH-01 tumours (Giordano et al. 2009).

The signalling pathways regulated by cardiac β -adrenergic and dopamine receptor signalling were over-represented. It is not known whether these GPCR are coupled to steroidogenesis in AIMAH-01. There was no cortisol response to β -adrenergic stimulation in the only patient available for such evaluation. Evaluation for an aberrant response to dopamine agonists is not part of the recommended screening protocol and was not examined (Lacroix 2009). We found discordant enrichment for genes regulated by ACTH in murine Y1 adrenal cells, consistent with the prevailing concept of the ACTH independence of tumorigenesis in AIMAH (Antonini et al. 2006).

DEG functions included cellular growth, proliferation, signalling and interaction. Some of the most highly DEG in our dataset have known roles in tumorigenesis and metastasis (e.g. myosin heavy chain 11, smooth muscle, *MYH11*; matrix metalloproteinase 16 (membrane inserted), *MMP16*; hyaluronan synthase 2, *HAS2*); some of these were also recently reported as DEG in sporadic AIMAH (Almeida et al. 2011). MARA suggested that upregulation of *MMP16*; *HAS2* may be due to increased activity of their transcriptional regulators.

We found enrichment for DEG in adrenocortical carcinoma (ACC) compared with normal adrenal gland/cortex or adrenocortical adenoma and for ACC with an aggressive, malignant clinical course (Giordano et al. 2009). Overall, most of the DEG and the GSEA data, the latter derived from integrated comparisons of our transcriptome data with previously published DEG sets, suggest that DEG are shared between AIMAH and malignant adrenocortical tumours. Our findings are substantiated by data from other studies. Several differentially expressed



microRNAs in sporadic AIMAH were previously implicated in tumorigenesis or metastasis (Bimpaki *et al.* 2010). In another study, gene expression profiling of several AIMAH nodules from one patient identified aberrant expression of oncogenic pathways in larger nodules (Almeida *et al.* 2011). These observations are intriguing given the benign biology of AIMAH, without propensity for metastasis. We therefore surmise that other genetic changes in target or supporting cells are not conducive to malignancy.

AIMAH tumours are regarded benign as metastases have never been documented clinically in the long-term post-operative surveillance of patients (Lacroix 2009). Whether subclinical metastases occur is unknown, as radiological surveillance is not performed. If AIMAH cells did have metastatic potential, then due to inefficient steroidogenesis, which in a metastatic cell might be even more pronounced, several decades might be required before manifesting clinically with CS. This may never eventuate, due to the late initial age of onset of AIMAH.

We found enrichment for DEG in sporadic AIMAH with mixed or no aberrant cortisol responses, suggesting that DEG are not specific to the aberrant receptor(s) being expressed and that molecular mechanisms may be shared between sporadic and familial AIMAH tumours – a novel concept as regards AIMAH. We did not identify mechanisms underlying VP receptor overexpression. However, glucocorticoids may upregulate *AVPR1A*, and VP may be involved in the paracrine regulation of cortisol secretion (Perraudin *et al.* 1993). Thus, ectopic overexpression of *AVPR1A* in AIMAH-01 may be secondary to tumorigenesis and hypercortisolism; the augmented *in vivo* cortisol response to VP may reflect the normal coupling of (overexpressed) *AVPR1A* to steroidogenesis. The mechanism of ectopic *AVPR1B* expression has not been elucidated.

The limitations of this study are the small numbers of samples evaluated and that we performed a ‘whole tissue’ gene expression study. AIMAH exhibits cellular heterogeneity and there are likely to be DEG

Figure 2 Heat map of 50 most upregulated (according to fold change) and 50 most downregulated genes in the AIMAH-01 tumours compared with normal adrenal cortex. The expression values of the probe sets were adjusted so that each probe set had the same mean expression value. Red indicates expression above the mean expression value for all probe sets; green indicates expression below the mean expression value for all probe sets. Normal: N126 and N134; AIMAH-01: III-1, III-2 and III-3; N1, 2: nodule 1, 2. This figure was generated in Multiexperiment Viewer program.

between cell types (e.g. steroidogenic enzymes in clear and compact cells) that may be diluted by studying the whole tissue, without having performed cellular partitioning. Partial characterisation of aberrant receptor expression by us and others in AIMAH is another limitation. However, complete characterisation of aberrant GPCR expression is not possible given that novel GPCR continue to be identified (Assie et al. 2010).

Ours is the first study to profile the transcriptome of familial AIMAH tumours. Using novel analytical approaches, we have identified involvement of pathways in familial AIMAH that have been implicated in sporadic AIMAH or other adrenocortical tumours, suggesting that mechanisms of tumorigenesis are shared between familial AIMAH and other sporadic adrenocortical tumours. This is a novel concept. We also found novel mechanisms of inefficient steroidogenesis in the familial AIMAH tumours. Our data are based on a small number of tumours studied, thus additional transcriptome studies of familial AIMAH are required. Ultimately, the identification of the genetic basis of familial AIMAH should enable further progress towards understanding the pathogenesis of these hereditary adrenocortical tumours.

Lucia Gagliardi^{1,2}

King-Hwa Ling³

Chung H Kok⁴

Joseph Carolan⁵

Peter Brautigan⁵

Rosalie Kenyon^{6,7}

Richard J D'Andrea^{2,4,7,8,9}

Mark Van der Hoek^{6,7}

Christopher N Hahn^{2,5}

David J Torpy^{1,2}

Hamish S Scott^{2,5,7}

¹Endocrine and Metabolic Unit, Royal Adelaide Hospital, Adelaide, South Australia 5000, Australia

²School of Medicine, University of Adelaide, Adelaide, South Australia 5000, Australia

³Department of Obstetrics and Gynaecology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
Departments of ⁴Haematology, ⁵Molecular Pathology, and ⁶Adelaide Microarray Centre, Centre for Cancer Biology, SA Pathology, Frome Road, Adelaide, South Australia 5000, Australia

⁷School of Molecular and Biomedical Science, University of Adelaide, Adelaide, South Australia 5000, Australia

⁸Centre for Stem Cell Research, University of Adelaide, Adelaide, South Australia 5000, Australia

⁹Haematology and Oncology Department, The Queen Elizabeth Hospital, Woodville, South Australia 5011, Australia

(Correspondence should be addressed to L Gagliardi who is now at Department of Genetics and Molecular Pathology, SA Pathology, Ground Floor, Frome Road, Adelaide SA 5000, South Australia, Australia; Email: lucia.gagliardi@health.sa.gov.au.)

Declaration of interest

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