Long noncoding RNA profiles of adrenocortical cancer can be used to predict recurrence

A R Glover1,*, J T Zhao1,*, J C Ip1, J C Lee3, B G Robinson1,2, A J Gill1,5, P S H Soon4,5 and S B Sidhu1,6

1Cancer Genetics Laboratory, Kolling Institute of Medical Research, Departments of 2Endocrinology 3Anatomical Pathology, Royal North Shore Hospital and University of Sydney, St Leonards, New South Wales 2065, Australia 4Department of Surgery, Bankstown Hospital and University of New South Wales, Bankstown, New South Wales 2065, Australia 5Ingham Institute for Applied Medical Research, Liverpool, New South Wales 2200, Australia 6University of Sydney Endocrine Surgical Unit, Royal North Shore Hospital, St Leonards, New South Wales 2065, Australia *(A R Glover and J T Zhao contributed equally to this work)

Correspondence should be addressed to S B Sidhu  
Email stanley.sidhu@sydney.edu.au

Abstract

Adrenocortical carcinoma (ACC) is an aggressive malignancy with high rates of recurrence following surgical resection. Long noncoding RNAs (lncRNAs) play an important role in cancer development. Pathogenesis of adrenal tumours have been characterised by mRNA, microRNA and methylation expression signatures, but it is unknown if this extends to lncRNAs. This study describes lncRNA expression signatures in ACC, adrenal cortical adenoma (ACA) and normal adrenal cortex (NAC) and presents lncRNAs associated with ACC recurrence to identify novel prognostic and therapeutic targets. RNA was extracted from freshly frozen tissue with confirmation of diagnosis by histopathology. Focused lncRNA and mRNA transcriptome analysis was performed using the ArrayStar Human LncRNA V3.0 microarray. Differentially expressed lncRNAs were validated using quantitative reverse transcriptase-PCR and correlated with clinical outcomes. Microarray of 21 samples (ten ACCs, five ACAs and six NACs) showed distinct patterns of lncRNA expression between each group. A total of 956 lncRNAs were differentially expressed between ACC and NAC, including known carcinogenesis-related lncRNAs such as H19, GAS5, MALAT1 and PRINS (P ≤ 0.05); 85 lncRNAs were differentially expressed between ACC and ACA (P ≤ 0.05). Hierarchical clustering and heat mapping showed ACC samples correctly grouped compared with NAC and ACA. Sixty-six differentially expressed lncRNAs were found to be associated with ACC recurrence (P ≤ 0.05), one of which, PRINS, was validated in a group of 20 ACCs and also found to be associated with metastatic disease on presentation. The pathogenesis of adrenal tumours extends to lncRNA dysregulation and low expression of the lncRNA PRINS is associated with ACC recurrence.

Key Words

- long noncoding RNA
- PRINS
- epigenetics
- recurrence
- metastasis
- microarray
- adrenocortical

Endocrine-Related Cancer  

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Introduction

Adrenocortical carcinoma (ACC) is an aggressive malignancy with limited treatment options and an overall survival of <35% (Abiven et al. 2006). ACC commonly recurs following surgical resection and the use of adjuvant mitotane is currently under investigation in the hope of reducing recurrence rates (http://clinicaltrials.gov, NCT00777244: Efficacy of Adjuvant Mitotane Treatment (ADIUVO); available at http://clinicaltrials.gov/show/NCT00777244, accessed 9/8/2014). Unfortunately, mitotane is associated with significant side effects, limiting its use, and a better understanding of ACC pathogenesis has therefore been identified as a key component in improving outcomes (Ronchi et al. 2014). Understanding of ACC pathogenesis is being greatly advanced by the advent of high-throughput genomic medicine, which has shown that ACCs have distinct genome-wide expression, microRNA expression and methylation profiles compared with adrenal cortical adenomas (ACAs) and normal adrenal cortex (NAC) (Giordano et al. 2009, Soon et al. 2009, Assie et al. 2012, Rechache et al. 2012).

An additional genomic marker of ACC pathogenesis could be long noncoding RNAs (lncRNAs). The discovery of the existence of thousands of lncRNAs and their diverse functions is causing a shift in the knowledge of biology (Cech & Steitz 2014, Morris & Mattick 2014). The lncRNAs are defined as RNA transcripts longer than 200 nucleotides that do not encode protein and can localise to the nucleus or cytoplasm (Li & Chang 2014). The expression of lncRNA is more tissue specific than protein-coding genes, suggesting that they have distinct functions in specific cells (Derrien et al. 2012). The functions of lncRNA include epigenetic silencing, transcriptional regulation, splicing regulation and acting as decoys for proteins and microRNAs (Morris & Mattick 2014). Furthermore, lncRNA dysregulation is being recognised as an important part in the development of cancer (Prensner & Chinnaiyan 2011) and recent exome sequencing and SNP array analysis has supported that lncRNA dysregulation may play a role in the pathogenesis of ACC (Assie et al. 2014). However, with these studies, lncRNAs have not been the intended target of the original analysis and as lncRNAs generally have a lower transcription abundance than other RNAs, they can be more difficult to detect using next-generation sequencing technology (Örom et al. 2010, Du et al. 2013).

Therefore, in this study, we performed focused lncRNA expression profiling using lncRNA microarrays to identify differentially expressed lncRNAs in ACCs compared with NAC and ACAs. We further investigated whether these lncRNAs expression levels are associated with ACC clinical outcomes such as recurrence. We have shown that multiple lncRNAs are dysregulated among ACC, NAC and ACA, and that individual lncRNAs expression levels can be associated with ACC outcome. This study identifies multiple potential diagnostic, prognostic and therapeutic targets for further functional study to better understand and improve outcomes in ACC.

Patients and methods

Patients and samples

Ethics approval was obtained from the Northern Sydney Area Health Service Human Research Ethics Committee and informed consent was obtained from all patients who participated in this study. Samples were obtained during surgery, snap frozen in liquid nitrogen and stored at −80 °C in the Neuroendocrine Tumour Bank of the Kolling Institute of Medical Research. The diagnosis of each sample was confirmed by an experienced endocrine pathologist (A J Gill) who was blinded to other data. Tumours with a Weiss score of <3 were classified as ACA and those with a Weiss score of ≥3 as ACC (Weiss 1984, Weiss et al. 1989). The ratio of tumour to non-neoplastic cells was calculated by histopathology and only samples with at least 80% tumour cells were included for analysis. NAC samples were obtained from non-paired adrenalectomy samples for non-functioning adrenal adenomas as described previously (Soon et al. 2009). The NAC was dissected by the operating surgeon who ensured that it was not adjacent to the adenoma and snap frozen. Histopathology was performed on the samples before analysis to ensure diagnosis of the samples and samples included only if they contained at least 80% NAC. Survival and recurrence outcomes were collated from the date of the surgery until a censor date of 1st May 2014. Recurrence was defined as a binary outcome (yes/no).

Tissue microarray

RNA extraction Total RNA was extracted from ~30 mg of fresh frozen tissue using a Qiazol protocol (RNeasy Mini Kit, Qiagen) according to the manufacturer’s instructions. RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Englewood, CO, USA) with a minimum RNA integrity number of seven required.
Microarray profiling

The ArrayStar Human LncRNA/mRNA Expression Microarray Version 3.0 (ArrayStar, Inc., Rockville, MD, USA) was used, which includes ~30,586 lncRNA probes and 26,109 mRNA transcripts and was constructed from six publicly available transcriptome databases and selected lncRNA publications (ArrayStar Human LncRNA Array V3.0, available at www.arraystar.com/microarray/service_main.asp?id=198, accessed 12/7/2014). In the microarray profiling, the RNA labelling, microarray hybridisation, slide washing and scanning were performed based on the standard protocols of ArrayStar. Acquired array images were analysed using the Agilent Feature Extraction Software (version 11.0.1.1). Quantile normalisation and subsequent data processing were performed using the GeneSpring GX Version 12.0 Software (Agilent Technologies). After quantile normalisation of the raw data, lncRNAs/mRNAs present in at least 15 out of 21 samples were chosen for further data analyses. A selection criterion of statistical significance calculated as a corrected P value of <0.05 using the Benjamini Hochberg false discovery rate method along with a minimum fold change cut-off of 2.0 (up- or downregulated) was applied to identify differentially expressed lncRNAs/mRNAs.

Quantitative reverse transcriptase-PCR

The expression levels of lncRNA were also measured by quantitative reverse transcriptase-PCR (qRT-PCR) using TaqMan lncRNA assays (Applied Biosystems). One microgram of total RNA of each individual sample was reverse transcribed using the High capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer’s instructions. Normalisation was performed by the global normalisation method or by endogenous control using the mRNA TaqMan probes of ACTB and GAPDH. The Applied Biosystems 7900HT Real-Time PCR System (Applied Biosystems) was used with standard TaqMan cycling conditions. All samples were run in triplicate. The relative expression (RQ) was obtained using the ΔΔCt method and the differences between groups were assessed statistically using DataAssist Version 3.01 (Applied Biosystems).

Microarray data

The microarray data used in this study are available from the Gene Expression Omnibus, series entry GSE61359.

Statistical analysis

For sample size calculation, a difference in lncRNA expression between controls (NAC) and study cases (ACC and ACA) of 30% was estimated. Using statistical significance of <0.05, a minimum of six cases were required for >90% power and a minimum of five cases for >80% power. For the analysis of clinically associated lncRNAs and clinical data, SPSS Statistics, release 21 (IBM Corp., Armonk, NY, USA) was used with a significance of <0.05 considered as statistically significant. Continuous variables were compared using the t-test or Mann–Whitney U test depending on distribution, and proportions of categorical variables of the clinical groups were compared using the χ2 test. For analysis of lncRNAs associated with outcome, calculations were performed using the GraphPad Software (La Jolla, CA, USA). Receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) were plotted using GraphPad. Survival estimates were calculated using the Kaplan–Meier estimate and compared using the log-rank test on two groups splitting by median expression into low- and high-expression groups.

Results

Patient samples and clinical characteristics

The lncRNA microarray included ten ACC (cases), five ACA (cases) and six NAC (controls) samples. Twenty ACC samples from the Kolling Institute Neuroendocrine Tumour Bank were used to validate the microarray findings and test lncRNAs associated with recurrence. The clinical characteristics and outcomes of the ACC patients included in this study are given in Table 1. There was no significant difference in ACC clinical characteristics between the microarray and validation group.

Table 1 Clinical characteristics of ACC patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Microarray (n=10)</th>
<th>Validation (n=20)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (range)</td>
<td>44.9 years (22.6–69.6)</td>
<td>45.4 years (17.8–75.1)</td>
<td>0.94</td>
</tr>
<tr>
<td>Mean Weiss score (range)</td>
<td>5.1 (3–9)</td>
<td>5.0 (3–9)</td>
<td>0.90</td>
</tr>
<tr>
<td>Tumour size (range)</td>
<td>8.8 cm (2.5–12.0)</td>
<td>7.8 cm (2.5–11.0)</td>
<td>0.44</td>
</tr>
<tr>
<td>Stage 4</td>
<td>33%</td>
<td>26%</td>
<td>0.94</td>
</tr>
<tr>
<td>Functional</td>
<td>50%</td>
<td>53%</td>
<td>0.90</td>
</tr>
<tr>
<td>Alive</td>
<td>80%</td>
<td>60%</td>
<td>0.44</td>
</tr>
<tr>
<td>Recurrence</td>
<td>30%</td>
<td>55%</td>
<td>0.77</td>
</tr>
<tr>
<td>Median follow-up (range)</td>
<td>1.4 years (0.1–4.2)</td>
<td>1.74 years (0.3–5.1)</td>
<td>0.37</td>
</tr>
</tbody>
</table>

All P values >0.05, referring to non-significant difference in clinical characteristics between each group.
Differential expression of lncRNAs in ACC compared with NAC

Microarray transcriptome analyses revealed a large amount of variation in lncRNA expression between ACC and NAC samples (Fig. 1A). Unsupervised hierarchical and heat map clustering showed systematic variations in the ACC samples compared with NAC samples with no samples incorrectly grouped (Fig. 1B). Nine hundred and fifty-six lncRNAs were differentially expressed in ACC compared with NAC, of which 476 were up-regulated and 480 were down-regulated ($P < 0.05$). The 956 differentially expressed lncRNAs corresponded to 809 described lncRNA genes. The percentages of lncRNA genes corresponding to differentially expressed lncRNAs by chromosome are

![Figure 1](http://erc.endocrinology-journals.org)

**Figure 1**
Differentially expressed lncRNAs between ACC and NAC. (A) Volcano plot of differentially expressed lncRNAs between ACC and NAC. Red plots represent RNAs with at least twofold change and corrected $P$ value $< 0.05$. (B) Unsupervised hierarchical clustering and heat map of lncRNA expression between ACC and NAC. Each column represents a sample and each row represents a gene. High relative expression is indicated in red and low relative expression in green. The bottom line represents the samples, with ACC represented in red (C) and NAC in blue (N). (C) Differentially expressed lncRNAs by chromosome for ACC and ACA compared with NAC. Percentage refers to proportion of lncRNAs differentially expressed for each chromosome by total amount of that group (ACC or ACA). Up-regulated lncRNAs are shown as a positive value and down-regulated lncRNAs as a negative value.
shown in Fig. 1C. The greatest difference in lncRNAs expression corresponded to chromosome 1 which amounted to 11.1% of lncRNAs differentially expressed between ACC and NAC.

Among the up-regulated lncRNAs, the maximum fold change was 30.7 for the lncRNA gene RP11-438N16.1. Among the down-regulated lncRNAs, the maximum fold change was 94.5 for the lncRNA gene RP11-64D22.2. A complete list of differentially expressed lncRNAs is attached separately as supplemental data (Supplementary Table 1a and b, see section on supplementary data given at the end of this article).

Notably, several lncRNAs, which have established functions in cancer development and cancer progression, such as growth specific arrest 5 (GASS), a tumour-suppressive lncRNA known to be down-regulated in breast cancer (Cheetham et al. 2013), was also found to be down-regulated in ACC. Other known cancer-related lncRNAs with differential expression included H19, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), psoriasis-associated RNA induced by stress (PRINS), guanine nucleotide-binding protein, alpha-stimulating-antisense 1 (GNAS-AS1) and maternally expressed 3 (MEG3) (Spizzo et al. 2012, Du et al. 2013). Array results of selected carcinogenesis-related differentially expressed lncRNAs between ACC and NAC are given in Table 2.

To test the validity of the microarray findings, we selected lncRNAs (CREM, PRINS, PHF14, MEG3, MALAT1, DLX6-ASI and LOC10013120) among the differentially expressed lncRNAs and analysed their expression by qRT-PCR. The validation cohort included 20 ACC samples and six NAC samples from the Kolling Institute Neuro-endocrine Tumour Bank.

MALAT1, DLX6-ASI and LOC10013120 were found in the microarray to be overexpressed in ACC (P ≤ 0.05). This was confirmed by qRT-PCR in the validation cohort, with these lncRNAs also overexpressed (P ≤ 0.05; Fig. 2). CREM and PRINS expression was down-regulated in ACC by microarray (P ≤ 0.05) and also by qRT-PCR (P ≤ 0.05) (Fig. 2). The microarray results of PHF14 and MEG3 were not confirmed in the validation cohort. There was increased expression of these lncRNAs in the microarray (P ≤ 0.05) while PHF14 and MEG3 were found to be non-significantly reduced by qRT-PCR (P = 0.57 and P = 0.92) in the validation cohort.

Differentially expressed lncRNA correlate with differentially expressed mRNAs

The array included probes for mRNAs as well as lncRNAs, which showed that 1523 mRNAs corresponding to 1341 protein-coding genes were significantly differentially expressed in ACC compared with NAC (Supplementary Table 2, see section on supplementary data given at the end of this article). To study correlation between differentially expressed lncRNAs and mRNAs, the Pearson correlation coefficients (PCC) were analysed for differentially expressed lncRNAs and mRNAs. Of the total differentially expressed 956 lncRNAs and 1523 mRNAs, 6396 matched lncRNA–mRNA pairs were identified with a PCC of > 0.9. These correlated pairs consisted of 393 individual lncRNAs and 453 individual mRNAs (Supplementary Table 3). Among correlated genes, 96.4% showed a positive correlation between mRNA and lncRNA expression. The lncRNA–mRNA co-expression network generated by Cytoscape (Smoot et al. 2011) is shown in Supplementary Fig. 1. The lncRNA–mRNA co-expression networks can serve as a prediction platform to investigate the potential function of the lncRNAs, particularly in this

**Table 2** Selected carcinogenesis-related differentially expressed lncRNAs between ACC and NAC

<table>
<thead>
<tr>
<th>Sequence name</th>
<th>Gene symbol</th>
<th>Regulation</th>
<th>P value</th>
<th>Fold change</th>
<th>RNA length</th>
<th>Chromosome</th>
<th>Strand</th>
<th>Relationship</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR_002819</td>
<td>MALAT1 (Lin et al. 2006)</td>
<td>Up</td>
<td>0.0057</td>
<td>4.19</td>
<td>8708</td>
<td>Chr11</td>
<td>+</td>
<td>Intergenic</td>
</tr>
<tr>
<td>NR_002785</td>
<td>GNAS-AS1 (Menigatti et al. 2013)</td>
<td>Up</td>
<td>0.0473</td>
<td>2.29</td>
<td>1158</td>
<td>Chr20</td>
<td>−</td>
<td>Intronic antisense</td>
</tr>
<tr>
<td>ENST00000524035</td>
<td>MEG3 (Zhou et al. 2012)</td>
<td>Up</td>
<td>0.0032</td>
<td>6.23</td>
<td>2853</td>
<td>Chr14</td>
<td>+</td>
<td>Intergenic</td>
</tr>
<tr>
<td>ENST0000437331</td>
<td>DLX6-ASI (Wahlestedt 2013)</td>
<td>Up</td>
<td>0.0242</td>
<td>2.40</td>
<td>580</td>
<td>Chr7</td>
<td>−</td>
<td>Intronic antisense</td>
</tr>
<tr>
<td>ENST0000439725</td>
<td>H19 (Gao et al. 2002)</td>
<td>Down</td>
<td>0.0178</td>
<td>25.31</td>
<td>1929</td>
<td>Chr11</td>
<td>−</td>
<td>Intergenic</td>
</tr>
<tr>
<td>ENST0000448718</td>
<td>GASS (Pickard &amp; Williams 2014)</td>
<td>Down</td>
<td>0.0070</td>
<td>2.54</td>
<td>565</td>
<td>Chr1</td>
<td>−</td>
<td>Intergenic</td>
</tr>
<tr>
<td>ENST0000443921</td>
<td>PRINS (Du et al. 2013)</td>
<td>Down</td>
<td>0.0056</td>
<td>5.59</td>
<td>2195</td>
<td>Chr10</td>
<td>+</td>
<td>Intron sense-overlapping Natural antisense</td>
</tr>
<tr>
<td>ENST0000495032</td>
<td>HOTAIRM1 (Zhang et al. 2009, 2014)</td>
<td>Down</td>
<td>0.0036</td>
<td>4.66</td>
<td>163</td>
<td>Chr7</td>
<td>+</td>
<td>Natural antisense</td>
</tr>
</tbody>
</table>
study, to identify the differentially expressed lncRNA involved in carcinogenesis or cell development. Examples of correlated lncRNA–mRNAs with possible functional roles in ACC tumourigenesis are given in Table 3.

### Differential expression of lncRNAs in ACC compared with ACAs

Eighty-five lncRNAs were differentially expressed ($P \leq 0.05$) in ACC compared with ACA (Fig. 3A), of which 37 were up-regulated and 48 were down-regulated (Supplementary Table 4, see section on supplementary data given at the end of this article). The 85 differentially expressed lncRNAs corresponded to 80 described lncRNA genes. Among the up-regulated lncRNAs, the maximum fold change was 47.2 for the lncRNA gene RP11-65L3.1. Among the down-regulated lncRNAs, the maximum fold change was 65.0 for the lncRNA gene RP11-64D22.2.

Despite a lesser difference in the total number of differentially expressed lncRNAs between ACC and ACA compared with ACC and NAC, unsupervised hierarchical and heat map clustering also showed systematic variations in the ACC samples compared with ACA samples with no samples incorrectly grouped (Fig. 3B).

### Differential expression of lncRNAs in ACAs compared with NAC

Between ACAs and NACs, 2655 lncRNAs were differentially expressed with 1999 lncRNAs up-regulated and 656 lncRNAs down-regulated (Supplementary Table 5, see section on supplementary data given at the end of this article). Among the up-regulated lncRNAs, the maximum fold change was 7.6 for the lncRNA gene RP11-65L3.1. The carcinogenesis-implicated lncRNA – MALAT1 (Lin et al. 2006) – was the second most up-regulated, with a fold change of 7.5. Among the down-regulated lncRNAs, the maximum fold change was 15.3 for the lncRNA gene RMST. The percentages of lncRNA genes corresponding to differentially expressed lncRNAs by chromosome are shown in Fig. 1C. The greatest difference in lncRNAs expression corresponded to chromosome 1, which

### Table 3  Examples of correlated lncRNA–mRNA with a potential functional role in ACC

<table>
<thead>
<tr>
<th>lncRNA gene</th>
<th>lncRNA sequence name</th>
<th>lncRNA array FC</th>
<th>mRNA</th>
<th>mRNA array FC</th>
<th>Correlation</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IKBKB</td>
<td>NR_033818</td>
<td>4.0 Up</td>
<td>IGFL2 (Entage et al. 2006)</td>
<td>3.8 Up</td>
<td>0.9604</td>
<td>Cell growth</td>
</tr>
<tr>
<td>PRINS (Du et al. 2013)</td>
<td>uc009skj.1</td>
<td>5.3 Down</td>
<td>WFDC1 (Madar et al. 2008)</td>
<td>15.9 Down</td>
<td>0.9325</td>
<td>Growth inhibitor</td>
</tr>
<tr>
<td>EMX2OS (Spiogoni et al. 2010)</td>
<td>uc001ldf.3</td>
<td>3.2 Up</td>
<td>DAXX (Assie et al. 2014)</td>
<td>3.1 Up</td>
<td>0.9411</td>
<td>Apoptosis regulation</td>
</tr>
<tr>
<td>RP11-181C3.2</td>
<td>ENST00000546421</td>
<td>2.5 Up</td>
<td>BUB1 (de Reynies et al. 2009)</td>
<td>7.2 Up</td>
<td>0.9115</td>
<td>Cell cycle regulation</td>
</tr>
<tr>
<td>H19 (Gao et al. 2002)</td>
<td>ENST00000439725</td>
<td>25.3 Down</td>
<td>SPON2 (Qian et al. 2012)</td>
<td>3.1 Down</td>
<td>0.9074</td>
<td>Neuron development</td>
</tr>
<tr>
<td>H19 (Gao et al. 2002)</td>
<td>ENST00000446406</td>
<td>15.3 Down</td>
<td>AADAC (Probst et al. 1994)</td>
<td>131.0 Down</td>
<td>0.9176</td>
<td>Carcinogen metabolism</td>
</tr>
<tr>
<td>ZEB2 EMX2OS (Spiogoni et al. 2010)</td>
<td>NR_033258 uc001ldf.3</td>
<td>4.2 Down</td>
<td>IGFBP9 (Rho et al. 2008)</td>
<td>5.8 Down</td>
<td>0.9041</td>
<td>Carcinogen metabolism</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.2 Up</td>
<td>VEGFB (Lautenschlaeger et al. 2013)</td>
<td>2.7 Up</td>
<td>0.9691</td>
<td>Cell signalling</td>
</tr>
</tbody>
</table>

FC, fold change of difference in array expression between ACC and NAC with corrected $P \leq 0.05$. 

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Figure 2  
QRT-PCR validation of lncRNA microarray analysis. FC, fold change in comparison of ACC vs NAC. *$ P \leq 0.05$ and **$ P \leq 0.01$ in comparison of ACC vs NAC, NS indicates $P$ value considered non-significant. Global normalisation method was used for analysis.
amounted to 9.2% of lncRNAs differentially expressed between ACA and NAC.

**Association of lncRNAs with ACC recurrence**

To identify lncRNAs associated with ACC recurrence, the lncRNA expression levels were compared between samples associated with and without recurrence. For this analysis, outcome data were available for nine of the ten patient ACC samples included in the microarray. This analysis identified 66 lncRNAs that were associated with recurrence (Supplementary Table 6, see section on supplementary data given at the end of this article).

Of these lncRNAs, the carcinogenesis-implicated lncRNAs GAS5, FEZF1-AS1, RPL23AP82, GNAS-AS1, MALAT1 and PRINS were selected to test this association with the 20 ACC samples of the validation cohort (Table 1). In this analysis, qRT-PCR confirmed PRINS to have a higher expression for non-recurrent ACC samples compared with recurrent ACC samples (FC=2.69, \( P=0.008 \)); however, the other five lncRNAs showed no significant difference between ACC with a clinical history of recurrence and that with non-recurrence (data not shown).

PRINS was found to have a decreasing level of expression among NACs, ACAs, ACC without recurrence and ACC with recurrence (Fig. 4), suggesting a possible role as a tumour suppressor. A low expression level of PRINS was also found to be associated with metastatic disease (\( P=0.030 \); Fig. 5A). To determine possible diagnostic accuracy of using PRINS as a marker of recurrence and/or metastatic disease, the AUC was determined (0.889, \( P=0.0044 \) for recurrence and 0.843, \( P=0.026 \) for metastatic disease; Fig. 5B). Using a sample splitting method, no difference was found in overall survival between high and low PRINS expression using the Kaplan–Meier method (\( P=0.17 \)).
Endocrine-Related Cancer

PRINS expression of the lncRNA possibility is emphasised by the discovery that low advantage in the management of adrenal tumours. This profiling could be used for diagnostic and therapeutic results indicate that lncRNA differential expression profile alone compared with both NACs and ACAs. These shows that the ACC can be classified by lncRNA expression expression levels by adrenal group. Normalised expression levels shown as median with error bars representing interquartile range, *P ≤ 0.05 and **P ≤ 0.01; rec, recurrence.

Discussion

This study is the first to demonstrate a distinct lncRNA expression profile distinguishing among ACC, ACAs and NAC. Unsupervised hierarchical and heat map clustering shows that the ACC can be classified by lncRNA expression profile alone compared with both NACs and ACAs. These results indicate that lncRNA differential expression profiling could be used for diagnostic and therapeutic advantage in the management of adrenal tumours. This possibility is emphasised by the discovery that low expression of the lncRNA PRINS is associated with recurrence in ACC.

PRINS was described in 2005 when it was found to be overexpressed in epidermal cells of psoriatic patients (Sonkoly et al. 2005). PRINS is located on chromosome 10, is over 2 kb bases long and consists of two exons. PRINS is transcribed by RNA polymerase II and is spliced, polyadenylated and contains a high density of stop codons and lacks an extensive open reading frame (Sonkoly et al. 2005). In epidermal cells, PRINS has been shown to regulate G1P3 (IFI6), an anti-apoptotic gene (Szegedi et al. 2010). Silencing of PRINS in HeLa cells has been shown to lead to altered cell morphology and up-regulation of multiple protein-coding genes, including Homo sapiens B-cell/lymphoma 9 (BCL9), which forms part of the Wnt/β-catenin signalling pathway (Mani et al. 2009, Szegedi et al. 2010). Abnormal activation of the Wnt pathway has been associated with poor outcome in ACC and further research is needed to clarify whether PRINS contributes to this activation in a tumour suppressor manner (Ragazzon et al. 2010).

New techniques to predict recurrence are essential for the management of ACC, due to the aggressiveness of this disease and propensity to recur. In current clinical practice, following primary tumour resection, recurrence rates can be predicted by use of the Ki-67 index (>10%) and other factors (Glover et al. 2013). PRINS expression levels could be included in this algorithm, allowing high-recurrence-risk patients to be recommended for adjuvant radiotherapy and mitotane and allowing low-risk patients to avoid these potential morbid treatments. The association of PRINS with distant metastatic disease is also of great clinical interest. As with many malignancies, the natural history of ACC is varied, with some patients having long disease-free intervals and many having short disease-free intervals of <6-12 months. These differences are probably due to the underlying tumour biology. The ability for clinicians to predict patients with metastatic disease allows for patients to be identified who may benefit from more intensive monitoring, avoid potential morbid local therapies and be offered clinical trials of novel systemic treatments.

Larger cohort studies examining the use of PRINS as a biomarker are required to assess this potential clinical application. Despite the advances in knowledge of the underlying molecular pathology of ACC and ACAs, the carcinogenesis pathway of ACC remains controversial.

Figure 4

PRINS expression levels by adrenal group. Normalised expression microarray levels of the IncRNA PRINS by clinical group. Expression levels shown as median with error bars representing interquartile range, *P ≤ 0.05 and **P ≤ 0.01; rec, recurrence.

Figure 5

PRINS expression levels in a validation cohort. (A) A comparison of the qRT-PCR lncRNA expression of PRINS in a validation cohort. Expression levels shown as median with error bars representing interquartile range, *P ≤ 0.05 and **P ≤ 0.01. The normalisation method used was endogenous control by ACTB and GAPDH, n = 19 for metastatic group due to stage at presentation not being available for one patient. (B) Receiver operating characteristic (ROC) curve for PRINS. Area under the ROC curve (AUC) = 0.889 for recurrence and AUC = 0.843 for metastatic disease.
compared with many other malignancies (Lerario et al. 2014). Molecular medicine supports that both carcinomas and adenomas develop in a similar way, with activation of abnormal pathways such as the Wnt pathway observed as an early change in development of both adenomas and carcinoma (Heaton et al. 2012). It is with interest that this study has demonstrated that the highest number of differentially expressed IncRNAs were between ACAs and NACs (2655 IncRNAs), while 956 IncRNAs were dysregulated between ACC and NAC, compared with 85 IncRNAs dysregulated between ACC and ACAs. These findings suggest that changes in IncRNA expression may be an early part in the pathogenesis of both ACC and ACAs.

This study has demonstrated that there are over 1000 IncRNAs differentially expressed in adrenocortical tumours, providing a base for functional research to elicit if these changes are due to driver mutations with underlying RNA regulatory function or due to a consequence of the neoplastic process (Morris & Mattick 2014).

The IncRNAs can be both tumour suppressive and oncogenic depending on the cell type as shown by H19 (Prensner & Chinnaiyan 2011). In ACC, H19 is thought to act as a tumour suppressor and H19 down-regulation is observed in ~90% of sporadic ACCs (Gicquel et al. 1997). In this study, H19 was reduced by a fold change of 25.3 in ACC compared with NAC.

The IncRNAs can act as a decoy for proteins. GAS5 binds directly to the DNA-binding domain of the glucocorticoid receptor, which allows it to act as a decoy, inhibiting glucocorticoid–regulated transcription in growth-arrested cells (Kino et al. 2010). As the glucocorticoid receptor is overexpressed in ACC (Tacon et al. 2009), down-regulation of GAS5 may play a role in this overexpression.

The IncRNAs can also act as translational regulators of mRNA (Morris & Mattick 2014). Our IncRNA–mRNA co-expression network analysis correlated a number of ACC-related genes that are of interest, including DAXX which has been recently discovered to be differentially expressed in a large series of ACCs analysed by exome sequencing and SNP analysis (Assie et al. 2012). DAXX was correlated with 41 different IncRNAs in our network, one of which, EMX2OS, has been shown to contribute to developmental regulation of transcription factors (Spigoni et al. 2010). Our co-expression network also identified other mRNAs of interest in ACC that were correlated with IncRNA expression including BUB1 (de Reynies et al. 2009), IGF2 (Emtage et al. 2006) and IGFBBP5 (Patel et al. 2014), which offers opportunity for further study to assess any functional relationship of this correlation.

Across all fields of molecular biology, due to the large number of IncRNAs identified, it is estimated that it will take a decade of analysis for specific IncRNA functions to be elicited (Cech & Steitz 2014).

The expression profiles of IncRNAs can distinguish among NAC, ACA and ACC. The IncRNA PRINS is a possible tumour suppressor in ACC. Lower expression levels of PRINS expression are associated with ACC recurrence and the presence of metastatic disease at presentation, identifying PRINS as a novel prognostic and therapeutic target for further investigation.


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