Abstract

MicroRNAs (miRs) are involved in the pathogenesis of several neoplasms; however, there are no data on their expression patterns and possible roles in adrenocortical tumors. Our objective was to study adrenocortical tumors by an integrative bioinformatics analysis involving miR and transcriptomics profiling, pathway analysis, and a novel, tissue-specific miR target prediction approach. Thirty-six tissue samples including normal adrenocortical tissues, benign adenomas, and adrenocortical carcinomas (ACC) were studied by simultaneous miR and mRNA profiling. A novel data-processing software was used to identify all predicted miR–mRNA interactions retrieved from PicTar, TargetScan, and miRBase. Tissue-specific target prediction was achieved by filtering out mRNAs with undetectable expression and searching for mRNA targets with inverse expression alterations as their regulatory miRs. Target sets and significant microarray data were subjected to Ingenuity Pathway Analysis. Six miRs with significantly different expression were found. miR-184 and miR-503 showed significantly higher, whereas miR-511 and miR-214 showed significantly lower expression in ACCs than in other groups. Expression of miR-210 was significantly lower in cortisol-secreting adenomas than in ACCs. By calculating the difference between dCT miR-511 and dCT miR-503 (delta cycle threshold), ACCs could be distinguished from benign adenomas with high sensitivity and specificity. Pathway analysis revealed the possible involvement of G2/M checkpoint damage in ACC pathogenesis. To our knowledge, this is the first report describing miR expression patterns and pathway analysis in sporadic adrenocortical tumors. miR biomarkers may be helpful for the diagnosis of adrenocortical malignancy. This tissue-specific target prediction approach may be used in other tumors too.

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Introduction

Sporadic adrenocortical tumors are common, and their prevalence reaches up to 7–9% in pathological series (Koch et al. 2002). Most of these are incidentally discovered, hormonally inactive benign tumors, whereas rare hormone-secreting adenomas (cortisol- or aldosterone-secreting) and malignancies of the gland are associated with significant morbidity and mortality. The prognosis of adrenocortical cancer (ACC) is poor with an overall 5 year survival below 30% (Libé et al. 2007).

The molecular pathogenesis of sporadic adrenocortical tumors is not well elucidated. Bioinformatic approaches including transcriptomics and comparative genome hybridization analyses have revealed several gene expression and chromosome alterations that appear to be relevant in adrenocortical tumor pathogenesis (Igaz et al. 2006). To our knowledge, no studies investigating potential differences between microRNA (miR) expression patterns of various sporadic adrenocortical tumors have been published to date.

miRs are small non-protein-coding RNAs involved in the post-transcriptional negative regulation of gene expression. Mature single-stranded miRs of ~22–24 nucleotides (Ambros et al. 2003) specifically bind to complementary sequences in 3'-untranslated regions of mRNA molecules (Chen 2005). miRs may induce translational repression or target mRNA degradation (Lim et al. 2005). Recent investigations on simultaneous miR-induced expression changes in transcriptome and proteome have revealed mRNA degradation as the principal way of miR action in mammalian organisms (Baek et al. 2008, Selbach et al. 2008). It has therefore been proposed that biologically relevant miR targets should be identified by the simultaneous analysis of miR and mRNA expression patterns without the necessity of investigating protein expression with high-throughput methods (Grosshans & Filipowicz 2008).

miR molecules are expressed in a tissue-specific fashion and may target various mRNAs in different organs. miRs have been implicated in the regulation of numerous cellular processes such as cell development, proliferation, differentiation, apoptosis (Wijnhoven et al. 2007, Bueno et al. 2008), and tumor development (Wijnhoven et al. 2007). Characteristic miR signatures were described in several non-endocrine (Barbarotto et al. 2008) and some endocrine tumors, e.g. follicular and papillary thyroid tumors (Weber et al. 2006), ovarian cancer (Yang et al. 2008), pituitary (Bottoni et al. 2007), and endocrine pancreatic tumors (Roldo et al. 2006).

The identification of potential miR targets is of pivotal importance for deciphering the molecular mechanisms of miR actions. Target selection can be performed by publicly available web-based databases using different computational target prediction algorithms. The most extensively applied databases include miRBase (Griffiths-Jones et al. 2008), TargetScan (Grimson et al. 2007), and PicTar (Krek et al. 2005). Since there is no unambiguous preference for any of these target prediction databases at present, target selection was performed arbitrarily by using a preferred algorithm in most studies. In silico target prediction algorithms take several biophysical, biochemical, and evolutionary parameters into consideration (Krek et al. 2005, Grimson et al. 2007, Griffiths-Jones et al. 2008); however, the predicted number of potential miR–mRNA interactions is generally huge and no data can be inferred on their biological relevance. Owing to the lack of objective target selection methods and the difficulties of experimental target validation, very few target mRNAs could be validated as post-transcriptionally regulated by specific miRs to date.

In this study, adrenocortical tumors have been examined by an integrative molecular and bioinformatic approaches including both miR and mRNA profiling. Since the selection of biologically relevant miR targets is unsolved at present, we introduced a novel, tissue-specific miR target prediction approach by using the results of a parallel transcriptomics analysis. In order to achieve tissue specificity, undetectable mRNAs were subtracted from the pool of potential miR targets and we searched for mRNAs with inverse expression alterations as their regulatory miRs. By this approach, the pool of potential miR targets can be reduced and biologically relevant targets may be selected. For this purpose, a novel data-processing software was developed (the experimental workflow is presented in Fig. 1). Combining these data with pathway analysis revealed important pathomechanisms involved in adrenocortical tumorigenesis.

Materials and methods

Tissue samples

Thirty-six tissue samples were studied: ten normal adrenocortical tissues (two males and eight females; mean age at surgery: 53.4 years), ten hormonally inactive adenomas (IA) (all females; mean age: 50.4 years), nine cortisol-producing adenomas (CPA) (two males and seven females; mean age: 40.16 years), and seven ACCs (two males and five females; mean age: 54.90 years) were studied. The study was...
approved by the Ethical Committee of the Hungarian Health Council, and informed consent was obtained from all patients involved. All ACCs were cortisol secreting. Normal adrenocortical tissues were obtained from patients undergoing total nephrectomy for kidney tumors. Tissue samples were immediately snap frozen in liquid nitrogen after removal and stored at \(-80\) °C until RNA isolation.

**miR expression profiling and quantitative reverse transcription-PCR**

Simultaneous miR and mRNA profiling was performed in 16 samples (four from each group). miRs showing significant differences in expression were examined in all tissue samples by quantitative reverse transcription-PCR (qRT-PCR).

Total RNA was isolated by TriReagent (Molecular Research Center Inc., Cincinatti, OH, USA) for miR expression profiling and qRT-PCR experiments. RNA integrity was verified by Agilent 2100 Bioanalyzer System (Agilent Tech. Inc., Santa Clara, CA, USA).

Total RNA of 100 ng per sample was reverse transcribed by TaqMan MicroRNA Reverse Transcription Kit and Multiplex RT for TaqMan MicroRNA Assays (all materials from Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instruction.

miR expression profiling of 368 miRs was performed on Taqman Low Density Array (TLDA) Human MicroRNA Panel v1.0 (Applied Biosystems) according to the manufacturer’s protocol using 7900HT Fast Real-Time PCR System (Applied Biosystems).

Normalized signal levels for each miR were calculated using comparative cycle threshold method (ddCT method; Livak & Schmittgen 2001) relative to \(RNU48\) following the manufacturer’s instruction.
(SDS programme, Applied Biosystems). Further data analysis was performed by BRB-ArrayTools Version 3.6.0 Stable Release (developed by Dr Richard Simon and Amy Peng Lam).

Differentially expressed miRs were identified by one-way ANOVA ($P<0.05$) using SPSS 15 for Windows statistical software (SPSS Inc., Chicago, IL, USA), followed by the conservative Scheffé post hoc test. In order to study only miRs of biological relevance further, by using an arbitrary filter criterion, miRs unexpressed in at least 50% of the samples studied by TLD Human MicroRNA Panel were filtered out.

Fourteen miRs with significant differences ($P<0.05$) in expression revealed by TLD Human MicroRNA Panel were selected for further validation and sample size extension by qRT-PCR. miRs with the most significant ($P<0.01$) differences in expression (10 miRs) and another four significant ($P<0.05$) miRs passing an arbitrary fold change filtering criterion (higher than threefold significant expression change) were selected for qRT-PCR analysis. Taqman MicroRNA Assays containing unique miR-specific RT primers were used as follows: miR-181b (4373116), miR-181d (4373180), miR-184 (4373113), miR-196b (4395326), miR-210 (4373089), miR-214 (4395417), miR-215 (4373084), miR-222 (4395387), miR-375 (4373027), miR-424 (4373201), miR-503 (4373228), miR-506 (4373231), miR-511 (4373236), miR-615 (4380991), and RNU48 (4373383) as housekeeping genes (all materials from Applied Biosystems). qRT-PCR was performed using TaqMan Fast Universal PCR Master Mix on a 7500 Fast Real-Time PCR System according to the manufacturer’s protocol. Data analysis was performed as above detailed using the SDS programme.

For qRT-PCR measurements, the same statistical analysis was performed as for the TLD Human miR Panel. In order to identify miR markers applicable for diagnosis, receiver-operating characteristics (ROC) analysis was performed by SPSS software.

**mRNA expression profiling**

Whole-genome mRNA expression analysis was performed on the same samples as used for miR profiling using 4×44 K Whole Human Genome Microarrays (Agilent Tech. Inc). Microarray experiments were done in a two-colour experimental design by comparing individual samples to a uniform reference pool of all samples examined.

For the mRNA microarray analysis, total RNA was isolated from homogenized tissue samples using RNeasy Lipid Tissue Mini Kit (Qiagen GmbH). RNA integrity was verified by Agilent 2100 Bioanalyzer System (Agilent Tech. Inc).

Total RNA of 1 μg mixed with RNA Spike-In Kit (Agilent Tech. Inc.) was reverse transcribed, and cyanine 5 or cyanine 3-CTP-labeled (Perkin-Elmer, Waltham, MA, USA). cRNA was synthesized using the Low RNA Input Linear Amplification Kit (Agilent Tech. Inc.) according to the manufacturer’s Dual Color Expression Microarray Protocol version 5.5. Successful labeling, cRNA yield, and purity were controlled on a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Cyanine 5-CTP-labeled cRNA of 825 ng per sample was mixed with an equal amount of cyanine 3-CTP-labeled uniform reference cRNA, and hybridized to 4×44 K Whole Human Genome Microarrays (Agilent Tech. Inc.) according to the manufacturer’s protocol.

Array scanning, feature extraction, and data normalization were performed with Agilent DNA Microarray Scanner and Feature Extraction Software 8.5 (Agilent Tech. Inc.) with default normalization scenario for Agilent 4×44 K two-colour array platforms.

Statistical analysis of mRNA microarray data was performed by Genespring GX 9.0 Software (Agilent Tech. Inc.) with default scenario. Identification of gene sets differentially expressed in microarray analysis was carried out by one-way ANOVA ($P<0.05$) followed by Benjamini–Hochberg multiple testing correction. Tukey’s Honestly Significant Difference test (Tukey’s HSD) was applied as standard multiple comparison test for microarray data analysis (Pavlidis 2003, Hulshizer & Blalock 2007).

**Tissue-specific miR target prediction**

Identification of potential miR target molecules was performed using TargetScan4.2 (http://www.targetscan.org), PicTar (http://pictar.org), and miRBase Targets Version 5 (http://microrna.sanger.ac.uk) databases.

The outputs of all three databases were merged and all potential targets of a miR predicted by these databases were identified by an own software application built with Microsoft Visual FoxPro 9.0 (Microsoft Inc.) developer system. This data-processing software is capable of converting the different target identifiers (HUGO Gene ID, Ensembl Transcript ID and RefSeq ID) applied by these prediction algorithms to each other. The annotation database was downloaded from the Ensembl homepage (www.ensembl.org). The software is able to identify...
all predicted target mRNAs for a miR or miR set, to provide score values retrieved from the different algorithms, and to mark mRNA targets overlapping in two or more databases.

As mRNAs not co-expressed with their regulatory miRs cannot be affected by miR regulation, these transcripts were filtered out from the list of potential targets by Microsoft Visual FoxPro 9.0. Arbitrary filtering criteria for subtracting absent mRNAs from microarray lists were the following: the percentage of ‘present flag’ and raw signal values higher than 50th percentile (of all raw signal values measured in the same sample) in less than three out of four samples in each experimental group. The mean of the 50th percentiles of raw signal values in the 16 samples studied considerably exceeded the background signal values.

We used Gene Set Enrichment Analysis (GSEA; Subramanian et al. 2005) to select potential mRNA targets with inverse expression alterations as their regulatory miRs. As a traditional gene expression analysis focuses on genes with significant differences in expression, biologically relevant, but fine alterations of gene expression data may remain unnoticed. GSEA is an alternative computational method developed for the analysis of these fine alterations. GSEA determines whether a priori or a user-defined set of genes shows statistically significant, concordant differences between two biological states (e.g. phenotypes). GSEA analyses gene expression data by rank statistics, and determines whether a particular set of genes is over- or underrepresented in the samples compared (Subramanian et al. 2005).

We used a novel GSEA approach to analyze potential, tissue-specific target sets of miRs with significant differences in expression alongside transcriptomic results, and to select potential mRNA targets with inverse expression alterations as their regulatory miRs. The enrichment analysis was performed by GSEA Software v2.0 (www.broad.mit.edu).

User-defined gene sets were created from tissue-specific filtered target lists of each miR with significant differences in expression in all pairwise comparisons of the groups studied. To reduce the amount of false-negative miR target interactions, a target set of a given miR contained those predicted tissue-specific targets, which were found to be expressed in at least one of the two compared experimental groups. Five miR target sets in normal versus ACC, four in IA versus ACC, four in CPA versus ACC, and one in normal versus CPA comparisons were established.

By GSEA, microarray data were rank ordered in a stepwise fashion based on differences in expression between the two groups compared. From the extremes (top or bottom: the most up- and downregulated mRNAs respectively) of these rank-ordered entire lists of microarray data, potential miR targets showing inverse expression alterations as their regulatory miRs could be selected. If a miR is underexpressed, its potential targets are supposed to be upregulated. Therefore, targets located at the top of ranked lists were selected by setting a cut-off at the maximal positive deviation of running enrichment score from zero. In a similar fashion, potential targets of overexpressed miRs are assumed to be downregulated, and therefore targets located at the bottom of the ranked list should be selected. This selection was performed by setting the threshold of the running enrichment score at the maximal negative deviation from zero.

Pathway analysis
GSEA-filtered tissue-specific miR targets and significant mRNA microarray results in all pairwise comparisons were analyzed using Ingenuity Pathway Analysis (IPA) 7.1 Software (Ingenuity Systems, Redwood City, CA, USA; www.ingenuity.com).

Results
miR expression analysis and biomarkers
Twenty-two miRs showed significant ($P<0.05$) differences in expression among the experimental groups studied by TLDA Human MiR Panel. mir-184 was detected only in ACC tissues. Both significant miRs and samples were then classified by hierarchical clustering based on similarities in expression profiles (Fig. 2).

Fourteen miRs were selected for further validation and sample size extension. By qRT-PCR, significant differences in expression were confirmed for six miRs (mir-184, mir-210, mir-214, mir-375, mir-503, and mir-511). The success rate for validating high-throughput TLDA measurements by qRT-PCR (6 out of 14 miRs) is similar to that previously reported by Resnick et al. (2009).

Significantly higher expression levels of mir-184 and mir-503 were detected in ACCs in comparison with their normal and benign counterparts, while mir-210 expression was significantly higher in ACCs compared with CPAs. Expression of mir-511 and mir-214 was significantly lower in ACCs relative to other experimental groups. On the other hand, expression of mir-375 was significantly higher in normal tissues compared with ACCs and CPAs (Fig. 3A and B).
To identify potential miR biomarkers of malignancy, miRs with the most significant delta CT (dCT) differences between ACCs and the other tissues (miR-184, miR-503, and miR-511) and the combination of these miR pairs were tested by ROC analysis (Fig. 3C). Setting the cut-off value of dCT $\Delta$miR-511–dCT $\Delta$miR-503 to 1.4 (diagnosis is ACC if the difference is $\geq 1.4$), ACCs can be identified with 100% sensitivity and 97% specificity. By setting the cut-off value of dCT $\Delta$miR-511 to 8.5, 100% sensitivity and 93% specificity could be achieved, while at a cut-off value of $-6.45$ for dCT $\Delta$miR-511–dCT $\Delta$miR-184, ACCs can be distinguished from benign samples with 100% sensitivity and 80% specificity.

Whole-genome mRNA expression profiling

Altogether 614 genes showed significant differences in expression among the groups studied ($P < 0.05$), significant microarray data are presented in the electronic Supplementary Table 1, which can be viewed online at http://erc.endocrinology-journals.org-supplemental/. In the ACC versus normal comparison, 302 genes were upregulated, whereas 301 were found to be downregulated in cancer tissues. All results are accessible at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo, accession number: GSE14922).

Several genes previously described as differentially expressed in already reported adrenocortical tumor microarrays have been revealed (Soon et al. 2008, Giordano et al. 2009). Topoisomerase II-α (TOP2A) expression was higher than ninefold in ACCs compared with other groups. Insulin-like growth factor 2 (IGF2) mRNA level was almost sevenfold higher in ACCs relative to IAs and threefold higher relative to CPAs and normal tissues. Overexpression of IGF2 is regarded as one of the most characteristic molecular alterations in ACC (Igaz et al. 2006, Soon et al. 2008, Giordano et al. 2009). Cyclin B2 (CCNB2) expression was at least 14-fold higher in ACCs than in any other tissue studied. Cell division cycle 2 and 25C (CDC2 and CDC25C) expression was also significantly higher in ACCs relative to the normal cortex and benign adenomas. On the other hand, cyclin-dependent kinase inhibitor 1C (CDKN1C) expression was significantly lower in ACCs relative to normal tissues.

Tissue-specific miR target prediction

Identification of miR target molecules was carried out using three web-based target prediction algorithms: TargetScan, miRBase, and PicTar. The outputs of these three algorithms were merged and analyzed by an own novel software application. The major advantage of our data-processing software is its potential for comparing the outputs of these algorithms, and identifying the overlapping targets predicted by two or more databases. Moreover, it provides an unfiltered list of all potential targets predicted by TargetScan, miRBase, and PicTar, which can be filtered further.

A total of 17 868 mRNA molecules have been predicted to be targets of the 6 miRs with significant differences in expression. Since one mRNA molecule can be targeted by more than one miR, this means only 13 147 different targets. Altogether, 7140 mRNAs were predicted by miRBase, 667 by PicTar, and 11 336 mRNAs by TargetScan. For the 6 miRs studied, 1275 overlapping targets could be identified, which amounts to 7% of all predicted targets (Table 1).

In order to reduce the false-positive predictions and to identify biologically relevant miR targets in adrenocortical tissues, we developed a novel tissue-specific target prediction approach by applying the results of the three target prediction algorithms and the simultaneously performed mRNA microarray
measurements. As mRNAs not co-expressed with their regulatory miRs cannot be relevant targets of miR-mediated regulation, gene products absent from the tissues studied were excluded from further analysis. The number of predicted miR targets could be considerably reduced by using the filtered sets of mRNAs (Table 1).

Recent reports (Baek et al. 2008, Grosshans & Filipowicz 2008, Selbach et al. 2008) have suggested that miR-evoked changes of protein level are well

Figure 3 qRT-PCR results of six miRs with significant differences in expression represented by ddCT values relative to the mean of normal samples (mean ± S.E.M). *P<0.05. (A) Significantly overexpressed, (B) significantly underexpressed miRs in ACCs. (C) ROC curves for the three most appropriate biomarkers for distinguishing ACCs from benign adrenocortical tumors.

Table 1 Numbers of potential targets for the six miRs with significant differences in expression predicted by TargetScan, PicTar, and miRBase and the number of tissue-specific targets expressed in human normal adrenal cortex, IA, CPA, and ACC samples

<table>
<thead>
<tr>
<th>miR ID</th>
<th>TargetScan</th>
<th>PicTar</th>
<th>miRBase</th>
<th>All predicted targets</th>
<th>Normal adrenal cortex</th>
<th>IA</th>
<th>CPA</th>
<th>ACC</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-184</td>
<td>609</td>
<td>17</td>
<td>1164</td>
<td>1683</td>
<td>771</td>
<td>906</td>
<td>905</td>
<td>909</td>
</tr>
<tr>
<td>miR-503</td>
<td>1547</td>
<td>NP</td>
<td>1225</td>
<td>2617</td>
<td>1359</td>
<td>1642</td>
<td>1635</td>
<td>1573</td>
</tr>
<tr>
<td>miR-511</td>
<td>3045</td>
<td>NP</td>
<td>1064</td>
<td>3945</td>
<td>2295</td>
<td>2615</td>
<td>2658</td>
<td>2536</td>
</tr>
<tr>
<td>miR-214</td>
<td>3874</td>
<td>528</td>
<td>1427</td>
<td>5252</td>
<td>2870</td>
<td>3463</td>
<td>3470</td>
<td>3367</td>
</tr>
<tr>
<td>miR-375</td>
<td>1790</td>
<td>107</td>
<td>1158</td>
<td>2871</td>
<td>1537</td>
<td>1781</td>
<td>1804</td>
<td>1744</td>
</tr>
<tr>
<td>miR-210</td>
<td>471</td>
<td>15</td>
<td>1102</td>
<td>1500</td>
<td>687</td>
<td>793</td>
<td>807</td>
<td>784</td>
</tr>
</tbody>
</table>

IA, hormonally inactive adenoma; CPA, cortisol-producing adenoma; ACC, adrenocortical carcinoma; NP, non-predicted.
reflected by mRNA expression alterations. mRNA degradation is thus considered as the principal way of miR action. To select the tissue-specific target mRNAs regulated by miRs mainly via target degradation, a novel GSEA approach was performed. By GSEA, we searched for target mRNAs showing inverse expression alterations compared with their potential regulatory miR molecules, as a further step to achieve tissue specificity. GSEA approach revealed 1306 targets for the significant miRs in normal versus ACC, 647 in IA versus ACC, 570 in CPA versus ACC, and 451 in normal versus CPA comparisons.

Pathway analysis
miR target sets revealed by GSEA in all pairwise comparisons and significant mRNA microarray data have both been subjected to IPA to identify potential pathogenetic pathways involved in adrenocortical tumorigenesis.

IPA revealed several possible pathways altered at the transcriptional level or potentially modified by miRs at the post-transcriptional level. Both IPA approaches (miR target mRNAs and gene transcripts with significant differences) in ACC to normal comparison revealed ‘cell cycle: G2/M DNA damage checkpoint regulation’ (Fig. 4, electronic Supplementary Tables 2 and 3, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/) as the top canonical pathway.

Discussion
miRs expressed in malignant tumors have been classified as tumor suppressors and oncogenes based on their relative under- or overexpression (Chen 2005). The overexpressed miRs in ACC (miR-503, miR-210, and miR-184) may thus be regarded as oncogenes, whereas the underexpressed (miR-511, miR-214, and miR-375) may be tumor suppressors in ACC.
Overexpression of miR-503 has already been described in human retinoblastoma samples relative to normal retinal tissues; however, its exact role in tumorigenesis is unclear (Zhao et al. 2009). Overexpression of miR-210 has been reported in breast cancer, and hypoxia may be a major factor in inducing miR-210 expression (Camps et al. 2008). Hypoxia is also a feature of malignant adrenocortical neoplasms (Bernini et al. 2002). miR-184 overexpression was noted in squamous cell carcinoma of the tongue (Wong et al. 2008).

miR-375 was found to be significantly underexpressed in both benign and malignant hepatocellular neoplasms associated with β-catenin mutations relative to their wild-type counterparts. Direct interactions between activated β-catenin and miR-375 are supposed (Ladeiro et al. 2008). Wnt–β-catenin signaling has been implicated in the pathogenesis of ACC (Soon et al. 2008); therefore, the underexpression of miR-375 in our ACC samples may support the relevance of this pathway. In both groups of cortisol-secreting tumors (CPA and ACC), low expression of miR-375 was found.

In vitro inhibition of miR-214 decreased, whereas inhibition of miR-210 increased apoptosis of HeLa cells (Cheng et al. 2005). miR-184 has also been supposed to act via the regulation of apoptosis (Wong et al. 2008). The relative overexpression of miR-210 and miR-184 and underexpression of miR-375 in our ACC samples may thus be associated with reduced apoptosis that is a common feature of malignancy. In human ovarian cancer, however, miR-214 was regarded as an oncogenic miR based on increased expression via targeting phosphatase and tensin homolog tumor suppressor gene (Yang et al. 2008). Furthermore, studies on human neuroblastoma tissues suggested that miR-184 may rather be proapoptotic (Chen & Stallings 2007). The same miR molecule may thus act both as an oncogene or a tumor suppressor and may have opposite actions in different tissues. These findings support the tissue-specific nature of miR action and the necessity of tissue-specific target prediction approaches.

As the histological diagnosis of ACC is often difficult, we tried to identify miR markers of malignancy. miR markers have already been shown to be applicable for the differentiation of benign and malignant tumors, e.g. follicular thyroid cancer (Weber et al. 2006). By ROC analysis, dCTmiR-511−dCTmiR-503 appeared to be the most appropriate marker of malignancy.

We used a novel tissue-specific target prediction approach for deciphering the biological relevance of the six significant miRs in adrenocortical tumors. This tissue-specific target prediction approach is based on the identification of all possible target mRNAs retrieved from three different target prediction algorithms and on the reduction of this huge target mRNA pool in two subsequent steps: i) subtraction of undetectable mRNAs based on microarray lists; ii) search for mRNA targets with inverse alterations in expression as their regulatory miR molecules. The database-processing software application used in our study was designed to retrieve all possible target mRNAs. The search for inverse alterations in expression was performed by the GSEA software.

Similar approaches for miR target identification have been published recently. Gennarino et al. introduced the software host gene oppositely correlated targets (HOCTAR) that analyzes mRNA profiling data, and looks for inverse expression correlations of genes hosting intragenic miR genes and potential miR target genes. In contrast with our approach, miR expression patterns are not directly analyzed by HOCTAR, and only the expression of miRs whose genes are localized within other genes can be studied (Gennarino et al. 2009).

Huang et al. developed the Bayesian data analysis algorithm, GenMiR++, which scores miR–mRNA pairs for inverse expression patterns. mRNA targets were predicted by TargetScan (Huang et al. 2007). Our approach also combines in silico target prediction algorithms with mRNA profiling; however, we used three target prediction algorithms in parallel and subtracted absent mRNAs from the pool of potential mRNA targets. Moreover, Huang’s algorithm searches for overexpressed miR and underexpressed mRNA pairs, whereas our approach also investigates pairs of underexpressed miR with overexpressed mRNAs.

Pathway analysis of both tissue-specific miR target mRNA sets and the significant mRNA microarray data revealed damage at G2/M checkpoint as a major pathogenic pathway in ACC. Cell cycle alterations including damage at G2/M have been described in the pathogenesis of several tumors (Molinari 2000), and there are some reports describing cell cycle activation in adrenocortical tumors too. The role of G1/S phase acceleration, overexpression of cyclin D1 (CCND1), loss of function of CDKN1C (p57kip2), and consequent overexpression of cyclin E (CCNE) and G1 cyclin-dependent kinases (CDK2 and CDK4) leading to cell cycle progress were found in sporadic ACCs (Bourcigaux et al. 2000, Lombardi et al. 2006). Cyclin B (CCNB1 and CCNB2), CDC2, CDC25B, CDC25C, and TOP2A involved in G2/M transition phase as cell cycle activators were overexpressed in our ACC samples. CCNB2 and TOP2A overexpression has already been described in ACCs (Giordano et al. 2009). CCNB/CDC2 complex was found to be overexpressed in...
several malignancies (Egloff et al. 2006); however, its exact role in ACC is unclear. Treatment of ACC cell lines either with 17β-estradiol (E_2) and progesterone (Brown et al. 2008) or with the adrenocortical compound mitotane combined with irradiation therapy (Cerquetti et al. 2008) induced G2/M block and apoptosis. Progesterone alone and in combination with E_2 induced mitotic perturbations in SW-13 cells via decreased CCNB1 and CCND1 expression (Brown et al. 2008). By activating cyclin-dependent kinase molecules, CDC25B is involved in the pathogenesis of several malignancies (e.g. hepatocellular carcinoma; Yan et al. 2008); however, there are no reports on its role in the pathogenesis of ACC.

In addition, underexpression of Reprimo (RPRM), which is regulated in a p53-dependent manner and is involved in cell cycle arrest at G2 (Taylor & Stark 2001), further supports that altered G2/M transition may be involved in the pathogenesis of ACC.

A few of these transcripts have been validated to be targets of miRs in non-adrenal cell lines (Mertens-Talcott et al. 2007, Bueno et al. 2008, Selbach et al. 2008). Based on our results, G2/M checkpoint regulation can thus be modified by miRs, and potential regulatory miRs in the adrenal cortex appear to be different from those validated in other tissues and cell cultures. However, it should be emphasized that miR target interactions reported in this study are only bioinformatically predicted interactions that should be further validated.

In conclusion, to our knowledge, this is the first report on miR expression signatures of sporadic adrenocortical neoplasms. We identified novel miR biomarkers suitable for distinguishing ACC from benign tumors with high sensitivity and specificity. As the histological diagnosis of adrenocortical tumors is often uncertain, these biomarkers can be of great help in cases where the assessment of malignancy is difficult. With our complex pathway analysis approach, different canonical pathways characterized by altered gene expression and potentially subjected to miR regulation were identified. The previously reported experimental data on the expression of cell cycle regulators and our finding that G2/M checkpoint damage has been revealed by both IPA approaches seem to support the feasibility of our tissue-specific target prediction approach.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Authors contribution statement**

P Igaz designed research; Z Tömöl, P M Szabó, V Molnár, Z Wiener, G Tölgyesi, and I Likó performed research; J Horányi and P Riesz supplied tissue samples; Z Tömöl, P M Szabó, V Molnár, P Reismann, A Patócs, R-C Gaillard, A Falus, K Rácz, and P Igaz analyzed data; and Z Tömöl, A Patócs, and P Igaz wrote the paper.

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