

Microarray gene expression and immunohistochemistry analyses of adrenocortical tumors identify IGF2 and Ki-67 as useful in differentiating carcinomas from adenomas

P S H Soon^{1,2}, A J Gill³, D E Benn¹, A Clarkson³, B G Robinson^{1,4},
K L McDonald¹ and S B Sidhu^{1,5}

¹Cancer Genetics, Kolling Institute of Medical Research, Royal North Shore Hospital, University of Sydney, St Leonards, Sydney, New South Wales 2065, Australia

²Department of Surgery, Bankstown Hospital and South West Clinical School, University of New South Wales, Sydney, New South Wales 2052, Australia

Departments of ³Anatomical Pathology, ⁴Endocrinology and ⁵Endocrine and Oncology Surgery, Royal North Shore Hospital, University of Sydney, Sydney, New South Wales 2065, Australia

(Correspondence should be addressed to P S H Soon; Email: patsysoon@med.usyd.edu.au)

Abstract

The management of adrenocortical tumors (ACTs) is complex. The Weiss score is the present most widely used system for ACT diagnosis. An ACT is scored from 0 to 9, with a higher score correlating with increased malignancy. However, ACTs with a score of 3 can be phenotypically benign or malignant. Our objective is to use microarray profiling of a cohort of adrenocortical carcinomas (ACCs) and adrenocortical adenomas (ACAs) to identify discriminatory genes that could be used as an adjunct to the Weiss score. A cohort of Weiss score defined ACCs and ACAs were profiled using Affymetrix HGU133plus2.0 genechips. Genes with high-discriminatory power were identified by univariate and multivariate analyses and confirmed by quantitative real-time reverse transcription PCR and immunohistochemistry (IHC). The expression of IGF2, MAD2L1, and CCNB1 were significantly higher in ACCs compared with ACAs while ABLIM1, NAV3, SEPT4, and RPRM were significantly lower. Several proteins, including IGF2, MAD2L1, CCNB1, and Ki-67 had high-diagnostic accuracy in differentiating ACCs from ACAs. The best results, however, were obtained with a combination of IGF2 and Ki-67, with 96% sensitivity and 100% specificity in diagnosing ACCs. Microarray gene expression profiling accurately differentiates ACCs from ACAs. The combination of IGF2 and Ki-67 IHC is also highly accurate in distinguishing between the two groups and is particularly helpful in ACTs with Weiss score of 3.

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Introduction

With the advent of better imaging methods and more widespread use of these techniques, adrenal tumors have been detected with increasing frequency. Adrenal tumors are common with a prevalence of adrenal incidentalomas in a recent computerized tomography series of 4.4% (Bovio *et al.* 2006). By contrast, adrenocortical carcinomas (ACCs) are rare with a

prevalence of 4–12/million population (NIH Consensus Statement 2002). Distinguishing between ACCs and adrenocortical adenomas (ACAs) can be difficult. The Weiss score, a nine-point histopathological scoring system, is presently the most widely used system for classifying adrenocortical tumors (ACTs) as benign or malignant. A total score of ≤ 2 is typically associated with a benign ACT while a score of ≥ 3 is indicative of malignancy (Weiss 1984, Weiss *et al.* 1989).

When Weiss first published in 1984, ACTs with a score of ≥ 4 were considered to be carcinomas (Weiss 1984). In 1989, this scoring system was revised so that ACTs with a score of ≥ 3 were considered to be carcinomas following the development of recurrence in a patient whose tumor had a Weiss score of 3 (Weiss et al. 1989). Consequently, ACTs with a Weiss score of 3 can be problematic with regard to diagnosis. The general aim of this study is to identify molecular markers that can robustly distinguish ACCs from ACAs, especially ACTs with a Weiss score of 3 where differentiation can be particularly difficult and inaccurate. We used microarray gene expression profiling to identify molecular markers which could accurately distinguish ACCs from ACAs. Expression profiles of a select group of genes were validated by quantitative real-time reverse transcription PCR (qPCR) and their clinical applicability tested using immunohistochemistry (IHC).

Materials and methods

Patients and tumors

Ethics approval for the study was obtained from the Northern Sydney Area Health Service Human Research Ethics Committee. Informed consent was obtained from patients prior to sample collection. Viable tumor tissue was surgically removed and snap-frozen in liquid nitrogen. The samples were stored at -80°C in the Neuroendocrine Tumor Bank of the Kolling Institute of Medical Research which contains tumors collected from 1992 to present. The tumors were reviewed by a single pathologist (A G) who was blinded to other data. Tumors with a Weiss score < 3 were classified as ACAs while those with a Weiss score ≥ 3 were considered to be ACCs. All except one ACC was primary tumor. Sample M5051 was a liver metastasis. Survival time (days) was counted from the date of operation. The census date for survival was July 31, 2007.

For the microarray, qPCR and IHC studies, a total of 28, 49, and 64 patients respectively were included. Patients with adrenal tumors as part of a known familial syndrome were excluded on clinical grounds. Six normal adrenal cortex samples were used for the microarray study while 2 and 15 were used for the qPCR and IHC studies respectively. Normal adrenal cortex was obtained from adrenalectomy samples away from the site of the tumor.

RNA extraction

Total RNA was extracted from fresh frozen tissue as previously described (Soon et al. 2008). Only samples with an RNA integrity number (Schroeder et al. 2006)

> 7.5 as determined on the Agilent Bioanalyser 2100 (Agilent, Santa Clara, CA, USA) were included in the microarray study. At the time that tumor samples were cut for RNA extraction, representative portions of the frozen tissue were extracted and then fixed and embedded in paraffin for histological assessment. A single observer, blinded as to other data, confirmed that the tissue consisted of either ACA or ACC and then calculated the ratio of tumor cells to non-neoplastic cells (e.g., lymphocytes, endothelial cells or non-neoplastic adrenal) in the processed tissue based on morphology. Only samples that were shown on histology to contain at least 80% tumor cells were included in this study.

Microarray preparation

Microarray gene expression profiling was performed on a cohort of 16 ACAs, 12 ACCs and 6 normal adrenal cortices.

The Affymetrix HGU133plus2.0 genechip that interrogates 54 675 genes was used in this study (Affymetrix, Santa Clara, CA, USA). cRNA conversion, hybridization, and scanning were performed at the Clive and Vera Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Sydney, Australia) and in accordance with the manufacturer's protocols. Normalization of the CEL files was performed with robust multiarray averaging utilizing R statistical software Version 2.3.1 (WU Wien, Wien, Austria) libraries contained in Bioconductor (<http://www.bioconductor.org/>).

Quantitative reverse transcription PCR

qPCR was used to quantify mRNA levels of *IGF2* (TaqMan Gene Expression Assay number Hs00171254_m1), *MAD2L1* (Hs00829154_g1), *CCNB1* (Hs00259126_m1), *ABLIM1* (Hs00242711_m1), *SEPT4* (Hs00365352_m1), *NAV3* (Hs00372108_m1), and *RPRM* (Hs00739877_s1) on a cohort of 29 ACAs and 20 ACCs (16 of the 29 ACAs and 11 of the 20 ACCs were also used in the microarray analysis), classified on Weiss score (Table 1). The gene expression pattern of these tumors was compared with a control sample consisting of two pooled normal adrenal cortices. A commercial total adrenal RNA (Ambion Inc., Austin, TX, USA) was also used as a reference. qPCR reactions for each sample were performed in triplicate in independent experiments. Total RNA (2 μg) was converted to cDNA using random hexamers (Invitrogen, Carlsbad, CA, USA) and Superscript III (Invitrogen) as previously described (Soon et al. 2008). All qPCR were performed using a 5' nuclease

Table 1 Patient demographics in the three groups

	Microarray study			qPCR study		
	ACCs N=12	ACAs N=16	Normal adrenal cortex N=6	ACCs N=20	ACAs N=29	Normal adrenal cortex N=2
Age (years) median (range)	52 (18–74)	48 (27–68)	60 (57–70)	40 (16–74)	51 (21–72)	59±2
Sex F:M	7:5	11:5	6:0	12:8	21:8	2:0
Presentation						
Incidentaloma (%)	5 (42%)	7 (44%)	N/A	5 (25%)	10 (34%)	N/A
Conn's syndrome (%)	0	7 (44%)	N/A	0	12 (41%)	N/A
Cushing's syndrome (%)	3 (25%)	2 (13%)	N/A	5 (25%)	6 (21%)	N/A
Virilization (%)	1 (8%)	0	N/A	2 (10%)	0	N/A
Hyperoestrogenism (%)	1 (8%)	0	N/A	2 (10%)	0	N/A
Mixed (%)	1 (8%)	0	N/A	2 (10%)	0	N/A
Other (%)	0	0	N/A	1 (5%)	1 (3%)	
Unknown (%)	1 (8%)	0	N/A	3 (15%)	0	N/A
Tumor size (mm)	87.5 (35–150)	28 (15–50)	N/A	94 (35–220)	25 (8–50)	N/A
Tumor weight (g)	125 (39–754)	22 (10–62)	N/A	240 (39–3600)	21.5 (7.2–75)	N/A
Weiss score	6 (3–9)	0 (0–2)	N/A	6 (3–9)	0 (0–2)	N/A
Outcome						
Alive without disease (%)	3 (25%)	16 (100%)	N/A	5 (25%)	29 (100%)	N/A
Alive with disease (%)	2 (17%)	–	N/A	3 (15%)	–	N/A
Dead of disease (%)	6 (50%)	–	N/A	9 (45%)	–	N/A
Unknown (%)	1 (8%)	–	N/A	3 (15%)	–	N/A

s.d., standard deviation; ACCs, adrenocortical carcinomas; ACAs, adrenocortical adenomas; qPCR, quantitative real-time reverse transcription PCR; N/A, field is not applicable.

technique with specific Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) and Taqman Universal PCR Master Mix, NO AmpErase UNG (Applied Biosystems) on a Rotorgene 3000 (Corbett Research, Mortlake, NSW, Australia). Ribosomal 18S RNA (Hs99999901_s1) was used as the endogenous control for normalization. Differences between groups were assessed statistically using Relative Expression Software Tool (REST-XL-version 2, Technical University of Munich, Munich, Germany; Pfaffl *et al.* 2002) where relative expression ratios are computed based on the PCR efficiency and crossing point differences.

Immunohistochemistry

IHC was performed on formalin-fixed paraffin embedded tissue sectioned at 4 µm onto positively charged slides (Superfrost plus, Menzel-Glaser, Germany). Paraffin blocks from a total of 79 specimens from the Department of Anatomical Pathology were available and these comprised 41 ACAs, 23 ACCs (14 of the 41 ACAs and 7 of the 23 ACCs were also used in the microarray analysis, and 20 of the 41 ACAs and 7 of the 23 ACCs were also used in the qPCR analysis) and 15 normal adrenal glands. The normal adrenal glands were obtained from nephrectomy specimens performed for renal or transitional carcinoma.

The primary antibodies and dilutions used in each case are listed in the Supplementary Table 1.

All slides were processed with the Vision Biosystems BondmaX autostainer (Vision Biosystems, Mount Waverley, Victoria, Australia). Specific processing details for each antibody are summarized in Supplementary Table 1, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>.

Antibody staining was interpreted in conjunction with a single haematoxylin and eosin (H&E) stained slide by a single pathologist (A G). At the time of examination, the pathologist was blinded as to the diagnosis and other data. However, the malignant or benign nature of many of the tumors was often apparent from the H&E stained slides.

Statistical analysis

Statistical analysis was performed with SPSS 12.0.1 (SPSS Inc., Chicago, IL, USA) for Windows and a $P < 0.05$ was considered significant. Categorical data were analyzed using Fisher's exact test. The Mann-Whitney test was used for qPCR statistical analysis because the data were not normally distributed and were heteroskedastic, despite attempted data transformation. The area under the receiver operating characteristic (ROC) curve was used to determine diagnostic accuracy of each of the six markers used in

IHC. The Spearman correlation coefficient was used to determine the degree of correlation between all six markers.

For microarray data analysis, both univariate and multivariate analyses were performed to detect differential expression between ACCs versus ACAs, ACCs versus normal and ACAs versus normal. Univariate analysis identified genes that were significantly differentially expressed between ACCs and ACAs. We used multivariate analysis as an additional method to allow us to compare the two lists of significantly differentially expressed genes and to select genes which were found to be overlapping from the two methods. We did this in order to identify the most significant and robust discriminators.

Univariate analysis, performed with affyImGUI analysis suite (Bioconductor) which uses a moderated *t*-statistic, was applied to the normalized data set using the Benjamini–Hochberg correction. Using a criteria of an *M*-value (\log_2 -fold change) of ≤ -2 or ≥ 2 and a *B*-statistic (log-odds expression) of ≥ 2 , the top 100 ranked differentially expressed gene lists were generated for ACCs versus ACAs, ACCs versus normal and ACAs versus normal. Data analysis was visually represented using Partek Genomic suite (Partek, St Louis, MI, USA). Two multivariate methods developed by the Australian Commonwealth Scientific and Industrial Research Organization, GeneRave and Stepwise Diagonal Discriminant Analysis (SDDA) were used to select genes with high-discriminatory power and were applied as previously described (McDonald *et al.* 2007).

Results

Patients for microarray analysis

The microarray analysis included 12 ACCs, 16 ACAs, and 6 normal adrenal cortices. Out of the 12 ACTs diagnosed histologically as ACCs using the Weiss score, eight have demonstrated local recurrence and/or metastases, three have remained disease free and one has been lost to follow-up.

Microarray analysis

Transcriptional profiles of the samples were generated using the Affymetrix HGU133plus2.0 genechips. Hierarchical clustering (Fig. 1A) generally showed that the gene expression patterns of normal tissue, ACAs, and ACCs were similar among tissues and tumors of the same type. One outlying normal sample (*, sample N3) and one outlying ACC (#, sample A1360) were observed. A heat map dendrogram that

was generated using the top 100 ranked significantly differentially expressed gene probes between ACCs and ACAs is shown in Fig. 1B. Using a cutoff of a *B*-statistic ≥ 2 and an *M*-value ≤ -2 and ≥ 2177 genes were found to be differentially expressed between ACCs and ACAs, 100 of which were upregulated and 77 downregulated. A summary of these genes is provided in Supplementary Table 2, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>. The multivariate analysis methods SDDA and GeneRave were applied to the data set to identify smaller sets of markers which could discriminate ACCs from ACAs. GeneRave identified 12 genes while SDDA identified nine genes (Supplementary Table 3, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>).

Interestingly, Acyl co-enzyme A dehydrogenase, very long chain (*ACADVL*) and Arachidonate 15-lipoxygenase, type B (*ALOX15B*), two genes located in a 10.4 Mb common minimal region of loss on the 17p13 locus in ACCs but not in ACAs, which we have previously found to be significantly downregulated in ACCs compared with ACAs by qPCR (Soon *et al.* 2008), were significantly differentially expressed in ACCs compared with ACAs on the microarray data. The respective *B*-statistic and *M*-value are 10.2 and -1.6 for *ACADVL* and 9.9 and -1.3 for *ALOX15B*.

Based on statistical significance of differential expression and *in silico* research, which revealed association of these genes with other cancers, nine genes from the univariate analysis list were selected for further study. *NAV3* from the SDDA list and *IGF2* from the GeneRave list were included in this list of nine genes. Both genes were significantly differentially expressed by univariate and multivariate analysis. In addition, *NAV3* was the only gene that was significantly differently expressed between the ACCs versus ACAs, ACCs versus normal and ACAs versus normal lists.

qPCR validation of genes

qPCR was used to quantify mRNA levels of *IGF2*, *MAD2L1*, *CCNB1*, *ABLIM1*, *SEPT4*, *NAV3*, and *RPRM* in 20 ACCs and 29 ACAs. Log mean expression of each gene for ACCs and ACAs is displayed in Fig. 2. The mRNA levels of all seven genes differed significantly between ACCs and ACAs ($P < 0.001$). Consistent with the microarray results, *IGF2*, *MAD2L1*, and *CCNB1* were significantly upregulated in ACCs compared with ACAs while *ABLIM1*, *NAV3*, *SEPT4*, and *RPRM* were significantly downregulated in ACCs compared with ACAs.

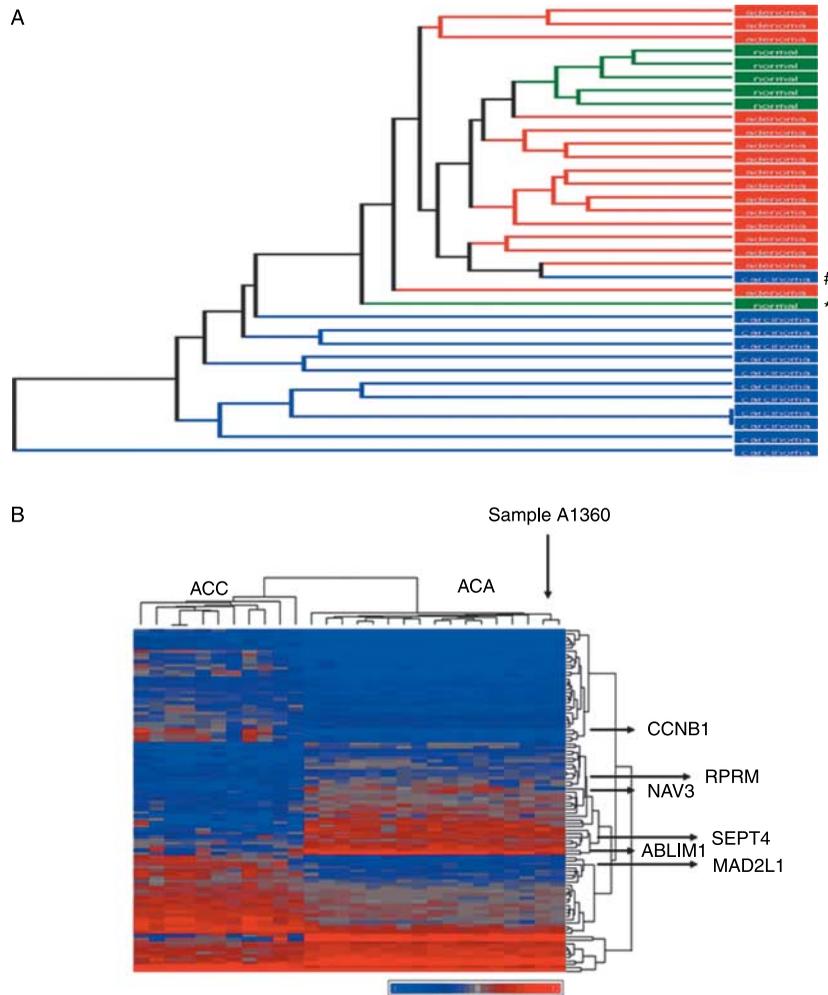


Figure 1 (A) Dendrogram from hierarchical clustering of the samples by gene expression. Samples in green are normal adrenal cortices; samples in red indicate ACAs while samples in blue are ACCs. #Indicates outlier ACC which clustered in with the ACAs. *Indicates outlier normal sample. (B) Heat map dendrogram generated from transcriptional profiles of 12 ACCs and 16 ACAs using the top 100 ranked significantly differentially expressed gene probes between ACCs and ACAs. Columns represent each sample while rows represent each gene. Sample A1360, classified as an ACC by Weiss score, has been grouped with the ACAs by this method of analysis. The location of the genes selected for further study by qPCR are shown except for *IGF2* which ranked 287th based only on the *B*-statistic and is not found in the top 100 significantly differentially expressed genes between ACCs and ACAs. There is heterogeneity of gene expression between the samples within each group.

Selection of genes for immunohistochemical analysis

Three genes, *IGF2*, *MAD2L1*, and *CCNB1*, from the microarray and qPCR studies were selected for IHC because they were significantly upregulated in ACCs compared with ACAs. *Ki-67* (a marker of proliferation which has been used clinically in the histopathological assessment of ACCs) IHC was also assessed. A further two genes, *ACADVL* and *ALOX15B*, which were found to be significantly downregulated in ACCs compared with ACAs by qPCR (Soon *et al.* 2008), were also selected for IHC analysis.

Immunohistochemical validation of genes

All 41 ACAs and 15 normal adrenal cortices were negative for IGF2, whereas 18 out of 23 ACCs (78%) were positive for IGF2 and tended to show definite perinuclear accumulation with or without significant cytoplasmic staining (score 2+ or more; Fig. 3A–C and Table 2).

Positive staining for MAD2L1 was typically observed in both the nucleus and cytoplasm. There was no significant staining observed in normal adrenal medullary, cortical tissues or normal adrenal glands. MAD2L1 staining was also absent in 39 of 41 (95%)

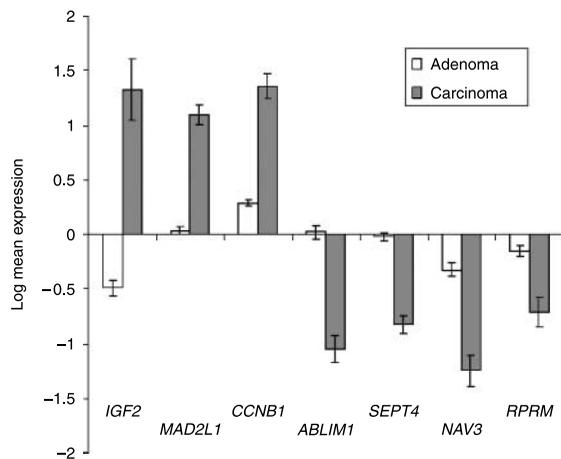


Figure 2 Quantitative real-time PCR results of the seven genes selected for validation shown as log mean expression in ACCs and ACAs. Comparison was made to a normal which comprised a pooled sample of two normal adrenal cortices. Error bars denote S.E.M. The mRNA expression of all seven genes differed significantly between ACCs and ACAs with $P < 0.001$.

ACAs. By contrast, 17 out of 23 (74%) ACCs were positive for MAD2L1 (Fig. 3D–F and Table 2).

CCNB1 cytoplasmic staining was negative in all 15 normal adrenal cortices and 41 ACAs. There was no significant staining in any normal adrenal medullary or cortical tissue although it was noted that occasional hematopoietic precursors as seen in incidental myelolipomas included a few positive cells. By contrast, CCNB1 demonstrated positive staining in 10 of 23 (43%) ACCs (Fig. 3G–I and Table 2).

For the marker Ki-67, all 41 ACAs, and 15 normal adrenal cortices showed a low proliferative index while 16 out of 23 (70%) ACCs demonstrated a high proliferative index (Fig. 3J–L and Table 2).

ACADVL staining was positive in 100% of ACAs and was also positive in the normal tissue but surprisingly, 15 of 23 (65%) ACCs were also positive for ACADVL (Fig. 3M–O and Table 2). All normal adrenal cortices and 33 of 41 (80%) ACAs were positive for ALOX15B and only 5 of 23 (22%) of ACCs were positive for ALOX15B (Fig. 3P–R). A unique pattern of ALOX15B staining was observed whereby the bulk of the tumor cells were negative and only scattered tumor cells displayed positive staining. This staining was predominantly in spindled cells around individual nests of tumor cells (a pattern reminiscent of the sustentacular cells of the adrenal medulla). Initially, we thought these cells may have been stromal, endothelial or sustentacular but additional IHC for S-100, CD31, CD34, Vimentin, calretinin, and Melan-A demonstrated that these cells were

compressed adrenal cortical tumor cells. Not surprisingly, loss of staining of these cells around tumor nests corresponded to a diffuse pattern of growth (one of the Weiss criteria for malignancy). The adrenal medullary cells were negative.

Diagnostic accuracy of IHC with each molecular marker was determined by area under the ROC curve. Figure 4 illustrates the ROC curve for all six markers. The area under the curve for IGF2, MAD2L1, CCNB1, and Ki-67 were all > 0.85 indicating high-diagnostic accuracy. On the other hand, the area under the curve for ACADVL was 0.311 and that for ALOX15B was 0.176 which signify poor diagnostic accuracy of these two markers.

Gene expression and IHC for individual samples gave similar results for IGF2, CCNB1, and MAD2L1. However, for ACADVL and ALOX15B, there was less correlation between gene expression and IHC, which would account for the low-diagnostic accuracy of these two markers.

Combined IGF2 and either Ki-67, MAD2L1 or CCNB1 IHC

Ki-67, MAD2L1, and CCNB1 are all markers of proliferation. The combination of positive staining for IGF2 and/or a high Ki-67 proliferative index identified 22 of 23 ACCs (96% sensitivity) but no ACAs (100% specificity). The sole ACC (tumor A1360) that was not identified by combination IGF2 and Ki-67 IHC had a Weiss score of 3 and clustered with the ACAs on microarray gene expression profiling. This tumor has not recurred or gone on to behave in a biologically malignant manner at 6 years follow-up indicating that it may represent a false positive of the Weiss scoring system.

The combination of positive staining for IGF2 and/or MAD2L1 identified all 23 ACCs (100% sensitivity) and only 2 of 41 ACAs (95% specificity). The combination of positive IGF2 and/or CCNB1 identified 21 out of 23 ACCs (91% sensitivity) and no ACAs (100% specificity). There was significant correlation between CCNB1, MAD2L1, and Ki-67 so that combinations of these markers without IGF2 did not increase the sensitivity or specificity.

Clinical follow-up data were available on 18 out of the 23 patients with ACCs. Correlation of clinical outcome of these patients with Weiss score and IGF2, MAD2L1, CCNB1, Ki-67, ACADVL, and ALOX15B IHC was performed and is shown in Supplementary Table 4, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>. This was conducted to determine if either the Weiss score or

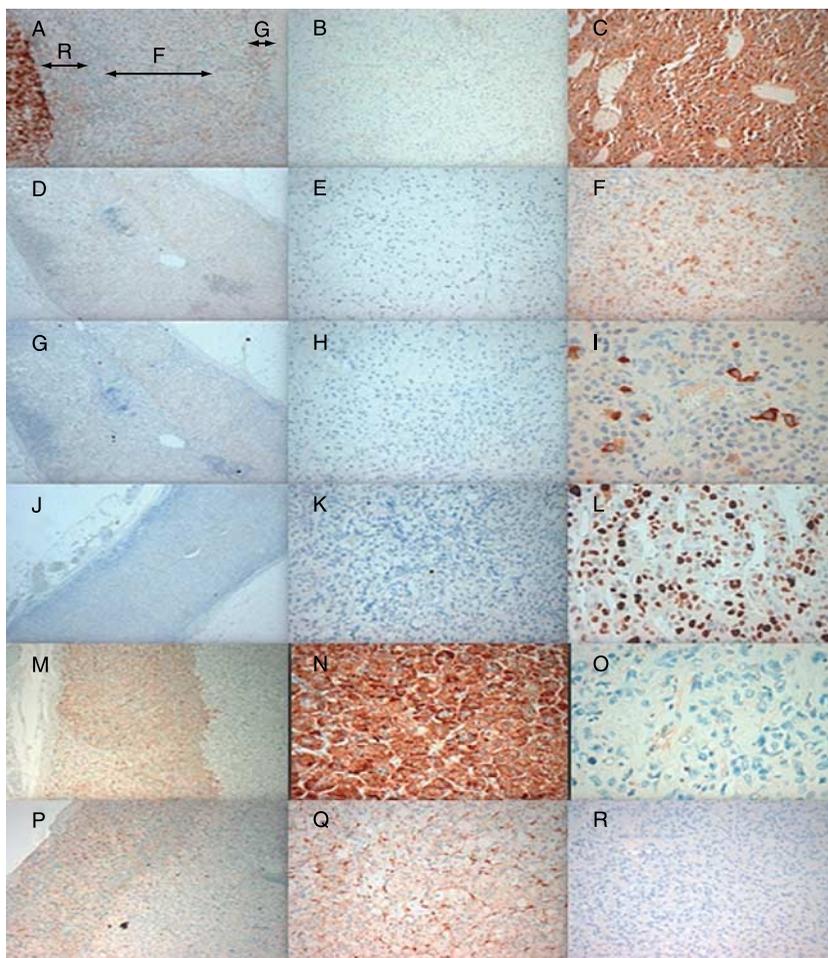


Figure 3 Representative micrographs of immunohistochemical staining with six molecular markers in normal adrenal, ACA, and ACC. Normal adrenals are to the left, ACCs are to the right and ACAs are in the middle. (A) IGF2 in the normal adrenal gland. The medulla (M) is diffusely strongly positive, the zona fasciculata (F) is negative whereas the zona reticularis (R) and glomerulosa (G) showed speckled cytoplasmic positivity without perinuclear accumulation (1+ staining) (original magnification 100×). (B) IGF2 in an ACA showing negative staining (score 0, original magnification 200×). (C) Positive IGF2 immunohistochemistry in an ACC (score 4, original magnification 200×). (D) MAD2L1 in the normal adrenal shows negative staining (original magnification 50×). (E) MAD2L1 in ACA showing negative staining (original magnification 200×). (F) Positive immunohistochemistry for MAD2L1 in ACC. MAD2L1 staining of the carcinoma cells was both nuclear and cytoplasmic (original magnification 200×). (G) Negative immunohistochemistry for CCNB1 in normal adrenal (original magnification 50×). (H) Negative CCNB1 immunohistochemistry in an ACA (original magnification 400×). (I) Positive CCNB1 immunohistochemistry in an ACC. CCNB1 staining in carcinoma cells was confined to the cytoplasm (original magnification 600×). (J) Immunohistochemistry for Ki-67 in the normal adrenal at low power. Only very occasional cells are positive and these are mostly lymphocytes (original magnification 50×). (K) Ki-67 in an ACA showing a low-proliferative index (original magnification 400×). (L) Ki-67 in an ACC showing a high-proliferative index (original magnification 600×). (M) ACADVL in the normal adrenal (original magnification 100×). (N) ACADVL in an ACA showing strong 4+ staining (original magnification 200×). (O) ACADVL in an ACC showing the absence of staining (score 0, original magnification 400×). Note that endothelial cells within the section act as positive internal controls. (P) ALOX15B in normal adrenal (original magnification 100×). (Q) ALOX15B in an ACA showing 3+ staining (original magnification 400×). Note the peculiar pattern of staining at the edge of nests. (R) ALOX15B in an ACC showing an absence of staining with a score of 0 (original magnification 200×).

IHC with the six markers would be able to predict clinical outcome of ACCs. It was noted that all the parameters examined did not correlate well with clinical outcome.

Clinical outcome was then correlated with Weiss score and IGF2 and Ki-67 IHC. All 41 tumors with Weiss score of 0–2 showed benign clinical courses and

all the tumors were negative for both IGF2 and Ki-67 IHC. Out of the nine patients with a Weiss score of 5–9, six had died of their disease, two were alive with disease and one was alive with no evidence of disease. All nine tumors were positive for either IGF2 and/or Ki-67. Three patients had a Weiss score of 4 and all were alive with no evidence of disease. All these

Table 2 Summary of immunohistochemistry results

		Normal adrenal N=15 (%)	ACA N=41 (%)	ACC N=23 (%)
IGF2	Negative score 0–1	100	100	22
	Positive score 2–4	0	0	78
MAD2L1	Negative < 10 cells stained/mm ²	100	95	26
	Positive ≥ 10 cells stained/mm ²	0	5	74
CCNB1	Negative < 10 cells stained/mm ²	100	100	57
	Positive < 10 cells stained/mm ²	0	0	43
Ki-67 labeling index	Low < 5% cells stained	100	100	30
	High ≥ 5% cells stained	0	0	70
ACADVL	Negative score 0–2	0	0	35
	Positive score 3–4	100	100	65
ALOX15B	Negative score 0–2	0	20	78
	Positive score 3–4	100	80	22

tumors were positive for either IGF2 and/or Ki-67. For the six patients with a Weiss score of 3, two had died of their disease. Both of these tumors were positive for either IGF2 and/or Ki-67. Out of the four patients who are alive with no evidence of disease, three tumors were positive for either IGF2 and/or Ki-67 while one tumor, the previously mentioned outlier tumor, was negative for both IGF2 and Ki-67, a staining pattern more consistent with ACAs.

Discussion

For pathologists, the Weiss score is routinely used to diagnose ACTs into ACC and ACA. However, when the tumor is borderline (Weiss score 3), the task of diagnosing an ACT as an ACC and not an ACA, remains problematic. We used gene expression profiling to identify robust biomarkers that can be used as an adjunct to the Weiss score to improve diagnostic accuracy.

Microarray gene expression profiling is a powerful tool that is able to characterize the transcription profile of a large number of genes in a tumor sample. In breast cancer, this method has been found to be more accurate than conventional histopathology in classifying tumors into different groups which correlated with clinical outcome (Perou *et al.* 2000, Sorlie *et al.* 2001). Hierarchical clustering grouped our samples on the basis of their gene expression and not on histological scoring. We found that it still tended to group the normal, ACA, and ACC samples together, showing good agreement with the original classification. However, two outlying samples were identified whose gene expression pattern did not conform with the others in the original histopathological classification. One ACC sample, A1360, was grouped with the ACAs while one normal sample was an outlier from the other normal samples (Fig. 1A). The outlier ACC sample was classified as an ACC based on a Weiss score of three but has behaved in a clinically benign manner to date, 6 years after the operation. It would therefore appear that this tumor is a false positive of the Weiss scoring system and that the tumor has been more accurately clustered with the ACAs by gene expression profiling. The other outlier, a normal adrenal cortex sample, was found upon review of histology to contain 20% medulla compared with <5% in the other normal samples. As the expression profile of the adrenal medulla is different to that of the adrenal cortex, the increased amount of medulla in this sample would explain the outlier status of this sample. This again demonstrates the ability of microarray gene expression profiling to distinguish samples which are dissimilar to the others, proving that it is a reliable and effective means of grouping similar samples together and differentiating ACCs from ACAs. It would therefore be a useful adjunct to the Weiss score.

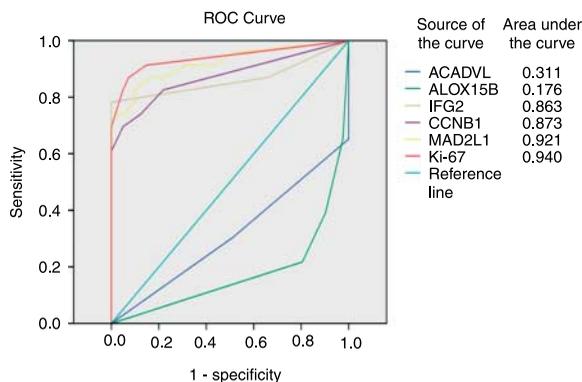


Figure 4 Receiver operating characteristic (ROC) curve for ACADVL, ALOX15B, IGF2, CCNB1, MAD2L1, and Ki-67 and values for area under the curve.

A total of five microarray gene expression studies have been performed comparing profiles of ACCs and ACAs, four with adult tumors (Giordano *et al.* 2003, de Fraipont *et al.* 2005, Velazquez-Fernandez *et al.* 2005, Slater *et al.* 2006), and one with pediatric tumors (West *et al.* 2007). Across all these studies including in our study, the only consistent finding has been the overexpression of IGF2 in ACCs compared with ACAs.

We selected nine genes from our microarray analysis for further study – five (*MAD2L1*, *CCNB1*, *ABLIM1*, *SEPT4*, and *RPRM*) were from the top 100 most significantly differentially expressed genes between ACCs and ACAs by univariate analysis, two (*IGF2*, *NAV3*) were from significantly differentially expressed gene lists by both univariate and multivariate analyses and two (*ACADVL*, *ALOX15B*) were from a region of loss of heterozygosity on 17p13 in ACCs which we previously reported (Soon *et al.* 2008) and found to be underexpressed in microarray analysis. *ACADVL* and *ALOX15B* are not listed in Supplementary Table 2 because their *M*-value was > -2 (-1.6 and -1.3 respectively). However, both genes have a significant *B*-statistic with values of 10.2 and 9.9 respectively. *IGF2* has been found in several studies to be overexpressed in ACCs compared with ACAs (Giordano *et al.* 2003, de Fraipont *et al.* 2005, Velazquez-Fernandez *et al.* 2005, Slater *et al.* 2006). *MAD2L1* (Tanaka *et al.* 2001), *CCNB1* (Wang *et al.* 1997, Soria *et al.* 2000, Hassan *et al.* 2001), *ABLIM1* (Kim *et al.* 1997), *SEPT4* (Elhasid *et al.* 2004, Gottfried *et al.* 2004), *RPRM* (Takahashi *et al.* 2005), and *ALOX15B* (Tang *et al.* 2002, Xu *et al.* 2003, Jiang *et al.* 2006, Wang *et al.* 2006) have all been associated with other cancers, while *ACADVL* (Gregersen *et al.* 2001) is associated with fatty acid metabolism which we hypothesized could be important in energy generation for cancer cells. The mRNA expression of these genes was significantly different between ACCs and ACAs, confirming the microarray gene expression results.

We studied five out of these nine genes, as well as Ki-67, by IHC to determine their clinical applicability. IHC was performed using antibodies to IGF2, *MAD2L1*, *CCNB1*, Ki-67, *ACADVL*, and *ALOX15B*. IGF2, *MAD2L1*, *CCNB1*, and Ki-67 each had an area under the ROC curve of > 0.85 which indicates high accuracy of each marker in differentiating ACCs from ACAs. It would appear somewhat contradictory that *CCNB1* with a sensitivity of only 43% but a specificity of 100% has an area under the ROC curve of 0.873. This is explained by the high specificity of *CCNB1*. Consequently, while

CCNB1 is not helpful in excluding an ACC when it is negative, a positive result in our study definitively indicates that the tumor is an ACC and would justify aggressive treatment. This marker is therefore useful if it is positive.

MAD2L1 was technically challenging to optimize for IHC because of its combined nuclear and cytoplasmic pattern of staining which mimics non-specific staining. While *CCNB1* was a less challenging stain to interpret, its results did not appear to add further value when compared with Ki-67 staining, which is an easier stain to score and is widely available. The expression of the *MAD2L1* and *CCNB1* proteins appeared to be focal in some ACCs. For instance, three out of the ACC samples had *MAD2L1* staining of 40 cells/mm² and one had *CCNB1* staining of 40 cells/mm² in small areas even though the majority of the tumor actually had an absence of staining. This would make scoring difficult and reproducibility less reliable if different sections of the tumor was stained and assessed. The area under the ROC curve for *ACADVL* and *ALOX15B* were low, with values of 0.311 and 0.176 respectively, indicating that these two markers are not helpful in differentiating ACCs and ACAs. However, the absence of staining for *ACADVL* is very specific for ACCs. Interestingly, even though the marker *ACADVL* had a low diagnostic accuracy with an area under the ROC of 0.311, it was highly specific for ACCs. All ACTs which did not stain for this marker were ACCs. *ACADVL* also had a low correlation with all the other markers on Spearman correlation, indicating that its mode of action is different to the other markers. In our cohort of ACCs, *ACADVL* did not identify any of the five ACCs that were not identified by IGF2. It did, however, identify two out of the seven ACCs which Ki-67 failed to identify. Consequently, it is possible to use this marker as an adjunct to the other markers. That is, if an equivocal result is obtained with the other markers, if there is negative staining with *ACADVL*, the tumor is highly likely to be an ACC.

Analysis with the Spearman correlation coefficient found high correlation between *MAD2L1*, *CCNB1* and Ki-67. All three markers play a part in the cell cycle and can be used as an indicator of proliferation. From our IHC study, in a clinical setting, we would conclude that the combination of IGF2 and a single proliferative marker, either *MAD2L1*, *CCNB1* or Ki-67, is highly sensitive and specific for ACC. Positive staining for IGF2 helps to identify the small proportion of carcinomas with low proliferative rates which go on to behave in a malignant manner. The combination of IGF2 and Ki-67 is much more feasible in a diagnostic

setting in preference to the combination with either CCNB1 or MAD2L1 which are more difficult to interpret. Several studies have found that IHC using Ki-67 is able to differentiate between ACCs and ACAs (Ino *et al.* 1997, Gupta *et al.* 2001, Giordano *et al.* 2003). While IGF2 mRNA overexpression has been noted in ACCs compared with ACAs by microarray gene expression analysis as well as quantitative PCR (qPCR; Giordano *et al.* 2003, de Fraipont *et al.* 2005, Velazquez-Fernandez *et al.* 2005, Slater *et al.* 2006), protein overexpression of IGF2 has also demonstrated with IHC (Erickson *et al.* 2001). Only one study has previously combined IGF2 and Ki-67 IHC. This study found a sensitivity and specificity of 100% and 95.5% respectively for differentiating ACCs from ACAs (Schmitt *et al.* 2006) which is similar to the 96% sensitivity and 100% specificity found in this study.

Conclusion

Whilst the Weiss score reliably classifies ACTs with a score of 0–2 as ACAs and those with a score of 4–9 as ACCs, the biological behavior of ACTs with a score of 3 can be difficult to accurately predict. Although Weiss score 3 ACTs are generally classified as ACC, many of them will be completely cured by surgery and therefore may represent false positives of the Weiss system. We have found that microarray gene expression profiling can accurately categorize ACTs into ACCs and ACAs. IHC using IGF2 and a marker of proliferation can also distinguish ACCs from ACAs. While microarray gene expression profiling also appears to accurately differentiate ACCs from ACAs, at present, because of its high cost, its usefulness in the clinical setting is limited. Consequently, for the group of ACTs with a Weiss score of 3, we would recommend the addition of IHC with IGF2 and a marker of proliferation (Ki-67 because of its wide availability and easy interpretation), in an attempt to more accurately classify and predict the biological behavior of these tumors. This would lead to expeditious treatment for patients with ‘true’ ACCs, avoid the unnecessary distress associated with a false positive diagnosis of ACC, and ultimately lead to better prognosis and improved quality of life.

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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