CLINICAL STUDY

Transcriptional profiling enables molecular classification of adrenocortical tumours

Cecilia Laurell*, David Velázquez-Fernández^{1,*}, Kristina Lindsten², Christofer Juhlin¹, Ulla Enberg¹, Janos Geli¹, Anders Höög³, Magnus Kjellman¹, Joakim Lundeberg, Bertil Hamberger¹, Catharina Larsson¹, Peter Nilsson and Martin Bäckdahl¹

School of Biotechnology, KTH-Royal Institute of Technology, 11427 Stockholm, Sweden, Departments of ¹Molecular Medicine and Surgery, ²Cell and Molecular Biology and ³Oncology-Pathology, Karolinska Institutet, 17176 Stockholm, Sweden

(Correspondence should be addressed to C Laurell; Email: clarell@kth.se)

*(C Laurell and D Velázquez-Fernández contributed equally to this work)

Abstract

Objective: Tumours in the adrenocortex are common human tumours. Malignancy is however, rare, the yearly incidence being 0.5–2 per million inhabitants, but associated with a very aggressive behaviour. Adrenocortical tumours are often associated with altered hormone production with a variety of clinical symptoms. The aggressiveness of carcinomas together with the high frequency of adenomas calls for a deeper understanding of the underlying biological mechanisms and an improvement of the diagnostic possibilities.

Methods: Microarray gene expression analysis was performed in tumours of adrenocortex with emphasis on malignancy as well as hormonal activity. The sample set consisted of 17 adenomas, 11 carcinomas and 4 histological normal adrenocortexes. RNA from these was hybridised according to a reference design on microarrays harbouring 29 760 human cDNA clones. Confirmation was performed with quantitative real time-PCR and western blot analysis.

Results: Unsupervised clustering to reveal relationships between samples based on the entire gene expression profile resulted in two subclusters; carcinomas and non-cancer specimens. A large number of genes were accordingly found to be differentially expressed comparing carcinomas to adenomas. Among these were *IGF2*, *FGFR1* and *FGFR4* in growth factor signalling the most predominant and also the *USP4*, *UBE2C* and *UFD1L* in the ubiquitin-proteasome pathway. Moreover, two subgroups of carcinomas were identified with different survival outcome, suggesting that survival prediction can be made on the basis of gene expression profiles. Regarding adenomas with aldosterone overproduction, *OSBP* and *VEGFB* were among the most up-regulated genes compared with the other samples.

Conclusions: Adrenocortical carcinomas are associated with a distinct molecular signature apparent in their gene expression profiles. Differentially expressed genes were identified associated with malignancy, survival as well as hormonal activity providing a resource of candidate genes for an exploration of possible drug targets and diagnostic and prognostic markers.

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Introduction

Adrenocortical tumours have a relatively high prevalence in the general population of up to 9% in autopsy studies (1, 2). However, malignancy is rare, the yearly incidence being 0.5–2 per million people, but with only 12 months mean survival time for patients with non-resectable cancers (3–7). The aggressiveness of carcinomas together with the high frequency of adenomas calls for a deeper understanding of the underlying biological mechanisms and an improvement of the diagnostic possibilities.

Most adrenocortical tumours are detected incidentally with computed tomography (CT) or magnetic resonance imaging (MRI), although some give clinical signs,

associated with the specific hormone over-expressed, e.g. aldosterone or cortisol. Adenoma development sometimes leads to overproduction of one or a mixture of these hormones. Aldosterone producing tumours (aldosteronomas) causes hypertension, while cortisol overproduction induces Cushing's syndrome. Adrenocortical carcinomas are often large and may therefore be present with abdominal pain and weight loss, with or without a mixture of the different hormones. The hormonal profiles associated with adrenocortical tumours makes hormonal screening an appealing clinical approach for diagnosis and analyses of steroid profiles may also contribute to set the malignant diagnosis. As a result of the recent advances in the bioimaging field and the more frequent use of CT and

MRI, the number of detected tumours is increasing. The main indication for adrenalectomy of tumours without hormone overproduction is the size of the tumour based on CT and MRI. All tumours with a diameter larger than 4 cm are, in most countries, recommended to be surgically resected (8). Development of adrenocortical carcinoma is not well characterised. Comparative hybridisation studies and allelotyping have shown that the number of genetic alterations increases with tumour size as a sign of an overall altered genetic stability in the malignant tumours (4, 5, 9). The most common genetic aberrations are gains on chromosome 4 and 5 and losses on chromosome 11 and 17. Also in benign tumours gains have been observed, on the long arm of chromosome 4 (10). Mutations of TP53 as well as over-expression of *IGF2* due to CpG island demethylation are shared features of malignant tumours (11).

Tumour classification based on microarray gene expression data has demonstrated promising results for numerous cancer types with potential clinical applications (12–15). There are principally two categories of classification: unsupervised and supervised, which are used for two different purposes. Unsupervised classification is mainly used for exploratory analysis, clustering tumours into groups based on similarities of gene expression profiles. If the resulting clusters have concordance with diagnosis or outcome the unsupervised clustering method can be used as a diagnostic and prognostic tool. Supervised classification on the other hand is always performed to create a diagnostic tool, which is to be used for discrimination of new data. Development of the classifier is based on prior information of how tumour types are connected to the appearance of gene expression profiles. This can be obtained by performing microarray analysis on samples with well-established diagnosis and with long follow-up.

In a previous microarray study (16), we have shown that adrenocortical carcinomas have gene expression signatures that can be distinguished from adenomas. In this report the sample size has been increased and we further analyse transcriptional differences in subtypes of hormone producing adrenocortical tumours, incidentalomas and normal adrenal cortex. Furthermore, based on the gene expression profiles, we suggest candidates for potential markers for the diagnosis of adrenocortical carcinoma. We also show that the carcinoma tumours cluster into two separate groups correlated to the survival time.

Methods

Tissue samples

This study included 4 normal adrenal cortex samples and all together 28 sporadic adrenocortical tumours; 4 aldosteronomas, 5 cortisol producing adenomas (here denoted as Cushing in figures and tables),

8 incidentalomas and 11 adrenocortical carcinomas. Cases with incidentaloma did not exhibit hormonal overproduction as determined by urinary and serum measurements. A subset of the tumours, 7 carcinomas and 13 adenomas, were included in a previous study and the data has been integrated into this extended study (16). All cases were selected from a cohort of 250 patients operated at the Karolinska University Hospital from 1986 to 2004 as described (16). Excluded cases were those with adrenal hyperplasia or tumours from the adrenal medulla. The tumour tissue analysed contained more than 70% tumour cells. Every patient had a clinical follow-up for at least 1.5 years. Diagnostic criteria for malignancy were vascular invasion, invasion of surrounding organs and/or presence of distant metastasis. In cases where these criteria were not fulfilled the histopathological criteria proposed by Weiss was used (17). Cases with high nuclear grade, increased mitotic figures and tumour necrosis were suspected to be malignant. The appearance of metastasis at follow-up, or recurrences further facilitated the establishment of a malignant diagnosis. Normal adrenal cortex samples were obtained from patients undergoing nephrectomy for other reasons and used as normal references in the RNA analysis. Matching normal adrenal cortex was obtained from four of the cases for western blot analysis. The clinical and tumour characteristics are shown in Table 1. The samples were obtained with informed consent and the study of the tissue material was approved by the Ethical Committee of the Karolinska University Hospital.

RNA extraction

A fresh frozen piece of ~100 mg was used for RNA extraction. Representative sections from all specimens were subjected to histopathological evaluation for tumour representativity. Total RNA was extracted using Qiagen mini and midi preps kits for RNA purification. RNA quantity and quality were assessed by an ND-1000 spectrophotometer (Nanodrop, San Francisco, CA, USA) and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Design of microarray experiments

We used a reference design where each sample was hybridised in duplicates. Tumour samples were labelled with Cy5 and hybridised with Cy3 labelled commercially available Universal Human Reference RNA (UHR; Stratagene, La Jolla, CA, USA).

Microarray production

In-house manufactured cDNA microarrays with 29 760 cDNA fragments representing 18 953 unique human UniGene IDs were used. The cDNA clones originate from the sequence verified human clone

Table 1 Clinical data of analysed cases.

Case no.	Histopathological diagnosis	Subtype	Age	Sex	Metastasis	Survival (months) ^a	Weight (g)	Size (cm)
50	Cancer	Cancer	63	F	Liver and bone	6 ^{DoD}	1500	20
372	Cancer	Cancer	78	M	No	3 ^{DoD}	203	15
882	Cancer	Cancer	72	F	No	5 ^{DoD}	85	7
1559	Cancer	Cancer	72	M	Lung	120	1036	11
1739	Cancer	Cancer	40	F	No	120	2350	13
1780	Cancer	Cancer	56	F	Liver and lungs	9 ^{DoD}	309	9
2582	Cancer	Cancer	54	F	Liver	4 ^{DoD}	1339	15
2716	Cancer	Cancer	68	M	No	69	1420	15
2931	Cancer	Cancer	52	M	No	67	660	11
3072	Cancer	Cancer	68	M	Liver	53	660	12
3410	Cancer	Cancer	84	F	Liver	32 ^{DoD}	1700	19
885	Adenoma	Cushing	38	F	–	151	12	3
1206	Adenoma	Cushing	63	F	–	146	51	6
3374	Adenoma	Cushing	66	F	–	50	14	2
4232	Adenoma	Cushing	35	F	–	20	26	4
4539	Adenoma	Cushing	42	F	–	18	28	4
501	Adenoma	Aldosteronoma	50	F	–	181	NA	2
1077	Adenoma	Aldosteronoma	47	M	–	144	NA	3
1105	Adenoma	Aldosteronoma	29	F	–	143	9	2
1196	Adenoma	Aldosteronoma	79	F	–	112 ^b	7	2
1180	Adenoma	Incidentaloma	63	F	–	140	36	5
1191	Adenoma	Incidentaloma	63	M	–	140	32	4
1764	Adenoma	Incidentaloma	42	F	–	110	17	3
2302	Adenoma	Incidentaloma	64	F	–	86	26	4
2414	Adenoma	Incidentaloma	63	F	–	84	22	4
2915	Adenoma	Incidentaloma	66	M	–	61	22	4
3669	Adenoma	Incidentaloma	54	F	–	31	29	4
3793	Adenoma	Incidentaloma	59	F	–	28	19	3
3234	Adrenal	Normal	81	M	–	–	–	–
4149	Adrenal	Normal	36	F	–	–	–	–
4166	Adrenal	Normal	85	M	–	–	–	–
5179	Adrenal	Normal	76	M	–	–	–	–

DoD, dead of disease.

^aTime between surgery and follow-up.^bDead.

collection, 97001.V, plates 1–310 (Research Genetics Inc., Huntsville, AL, USA). The microarrays were spotted onto Ultra GAPS slides (Corning, Lowell, MA, USA) with a Qarray2 arrayer with 48 K2805 pins (Genetix, Hampshire, UK). The complete protocols for preparation of clones and printing together with a complete gene list can be found at www.ktharray.se and at ArrayExpress (A-MEXP-114).

Target labelling

First strand cDNA target was generated by RT of 10 µg of total RNA in a 30 µl reaction using 5 µg random hexamers (Operon), first-strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂), 0.01 mM dithiothreitol (DTT), 400 units Superscript III (Invitrogen), 2 mM d(A/G/C)TPs, 1.6 mM dTTP (Amersham Biosciences) and 0.4 mM aminoallyl-dUTP (Sigma-Aldrich). Secondary structures of RNA and primers were removed at 70 °C during 10 min followed by 2 min of incubation on ice for primer annealing before addition of the other reaction components. cDNA synthesis was performed for 2 h at 42 °C and

terminated by addition of EDTA. The RNA was hydrolysed in NaOH during 15 min incubation at 70 °C followed by neutralisation with HCl (final concentrations 20, 150 and 150 mM, respectively). The cDNA was purified using MinElute spin columns (Qiagen) with the provided wash buffer replaced by 80% ethanol and the elution buffer by 100 mM NaHCO₃, pH 9.0. Monofunctional NHS-ester Cy3 or Cy5 fluorophores (Amersham Biosciences) were coupled to the amino-allyl groups during a 30-min incubation at room temperature after which unincorporated ester groups were inactivated through a hydroxylamine treatment (final concentration of 730 mM). The labelling reactions were purified using MinElute spin columns. The complete protocol for target labelling can be downloaded from www.ktharray.se. Extraction and labelling quality/quantity were assessed with an ND-1000 spectrophotometer (Nanodrop).

Hybridisations and scanning

The slides were prehybridised in 5 × SSC, 0.1% SDS and 1% BSA (Sigma-Aldrich) for 30 min in 42 °C. The

hybridisation buffer contained 50% formamide, 5×SSC, 0.1% SDS, 10 µg COT-1 DNA (Invitrogen) and 5 µg tRNA (Sigma–Aldrich). After hybridisations in chambers (Corning) for 18 h at 42 °C the arrays were washed according to Corning's recommendations and scanned at 532 and 635 nm with 10-µm resolution using a G2565BA DNA microarray scanner (Agilent Technologies).

Data analysis

Image analysis was performed in GenePix Pro 5.1 software (Axon Instruments Inc., Foster City, CA, USA). Spots were removed from further analysis if more than 70% of foreground pixels were below background intensity plus two s.d.s in both channels or if the difference between ratio of medians and regression ratio exceeded 20% in one of the channels. After removal of the low quality spots the remaining intensities were print tip lowess normalised in the R environment for statistical computing (18) with the aroma package (19). To increase robustness considering the limited amount of patients, probe signals were required to be present in at least one technical replicate for each patient to be considered in the further analysis (6063 probes representing 4843 unique UniGene IDs). An exception was made in the comparison of aldosteronomas to all other samples where a maximum of five missing values were allowed in non-aldosteronoma specimens (12059 probes representing 8880 unique UniGene IDs). An empirical Bayes statistics (B-test) for ranking differentially expressed genes according to statistical significance was performed with the Limma package (20). The criteria for statistical significance in the supplementary data files of differentially expressed genes were $P < 0.001$ and $\log_2$ fold change > 1 . The P value is the Benjamini and Hochberg false discovery rate. Fischer's exact test was used for enrichment analysis of Gene Ontology (GO) terms and chromosomal location among the 1000 most significantly

differentially expressed genes comparing malignant to non-malignant samples with a significance level of $P < 0.05$. Clustering was performed with the stats and class packages.

Quantitative real time-PCR

In order to confirm the array findings we used a two step TaqMan quantitative real time-PCR (qRT-PCR) analysis. Synthesis of cDNA from total RNA was performed using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) on 0.5–1 µg of RNA from each sample. The reaction was carried out in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems), under the conditions of an initial hold phase of 25 °C for 10 min, followed by 2 h at 37 °C.

A 96-well plate was prepared for the TaqMan Gene Expression Assays (Applied Biosystems). Every well contained 12.5 µl of TaqMan Universal master mix, 1.25 µl of primer plus probe mix (Assays on Demand from Applied Biosystems), 6.25 µl of RNase-free water and 5 µl of cDNA or previously prepared dilutions (reference curve). Dilutions were prepared for 1:10, 1:100, 1:1000 and 1:10 000. Every sample and control well was analysed in triplicates and the reference genes in duplicates according to manufacturer's recommendations. A non-template control well was used as negative control in every run. Wells were sealed with MicroAmp Optical caps. Plates were loaded into the ABI PRISM 7700 sequence detection system (Applied Biosystems). In order to select a reference gene for the quantification we used the TaqMan Human Endogenous Control Plate. This evaluates the expression of 11 selected reference genes, using qRT-PCR. The plate also features an internal positive control designed to detect the presence of PCR inhibitors in test samples. The explored genes were: 18S rRNA, acidic ribosomal protein, beta-actin, cyclophilin, GAPDH, phosphoglycerokinase, b2-microglobulin, beta-glucuronidase, hypoxanthine ribosyl transferase, transcription factor

Figure 1 Unsupervised clustering of gene expression profiles in the entire sample set based on all microarray features. Principal component analysis reveals sample similarities and shows that the gene expression is tumour type specific (left). The same observation can be made with hierarchical clustering using Euclidean distances and average linkage (right).

IID TATA binding protein and the transferrin receptor. The 18S rRNA gene was found to have the most stable expression levels among all samples and was thereby chosen as the reference gene to relate the transcript levels of the target genes. The genes analysed with qRT-PCR were: aldehyde dehydrogenase 1 family member A1 (*ALDH1A1*), insulin-like growth factor II precursor (*IGF2*), ubiquitin specific peptidase 4 (*USP4*) and ubiquitin fusion degradation protein 1 (*UFD1L*).

Western blot analysis

Frozen tissues were ground into powder by mortaring on dry ice and subsequently dissolved by sonication in a buffer containing 50 mM Tris (pH 7.4), 5 mM MgCl₂, 250 mM sucrose, 1 mM DTT and 1 mM ATP. The protein extracts were clarified by centrifugation and the lysates were snap-frozen in liquid nitrogen and kept frozen until use. For western blot analysis 20 µg protein/sample was fractionated on SDS-PAGE 4–12% gradient gels (Invitrogen), blotted on PVDF membranes, blocked in PBS supplemented with 5% skim milk and 0.1% Tween and probed with anti-Usp4 (C-term) antibody (Abgent Inc., San Diego, CA, USA), diluted 1:500. After subsequent washings and incubation with HRP-conjugated goat anti-rabbit serum (Amersham Biosciences), the blots were developed by ECL (GE Healthcare, Uppsala, Sweden). As a positive control for the Usp4 antibody we transfected human embryonic kidney 293T cells with a plasmid expressing USP4 in fusion with green fluorescent protein and harvested the cells for western blot analysis two days post-transfection.

Results

Classification with unsupervised methods

Principal component analysis, hierarchical clustering, *k*-means clustering and self-organising maps were applied to the filtered set of 6063 probes with expression values obtained in every tissue sample analysed. The carcinomas and the non-cancers clustered in separate groups with the exception of carcinoma 2931 (Fig. 1). The discrepancy in gene expression is striking and the malignant behaviour seems to be congruent and out-rule other inter-individual differences. Interestingly, the adenoma cluster fell into subgroups, with all incidenta-lomas represented in one group, all aldosteronomas in a second group and normal cortex and Cushing adenomas mixed into a third.

Differential gene expression in cancers

A *t*-test moderated with an empirical Bayesian statistics was applied to test for differential expression. When carcinomas, except case 2931, were compared to all

other samples using the criteria for statistical significance described in Materials and methods, a significantly differential expression was observed for around 300 transcripts, Supplementary file 1A, which can be viewed online at <http://www.eje-online.org/supplemental/>. The 50 most significantly overexpressed and the 50 most significantly underexpressed transcripts are displayed in Fig. 2. In Supplementary file 1B statistical data for all genes present in all samples is displayed. *IGF2*, a well established gene overexpressed in

Figure 2 Expression profiles of the 50 most significantly up-regulated (top) and down-regulated (bottom) genes are displayed comparing cancer patients to non-cancer patients. The colour scale ranges from red to green analogous to over- to underexpression. In Supplementary files 1A and B, fold changes and *P* values are given.

Table 2 Genes with significant differential expression in carcinomas versus non-malignant samples in the Gene Ontology categories of adhesion, mitosis and extracellular location.

Accession	UG cluster	Gene name	Symbol	Log ratio
Adhesion				
AA455997	Hs.75081	Adenomatosis polyposis coli	APC	0.79
AA034051	Hs.75081	Adenomatosis polyposis coli	APC	1.05
AA490462	Hs.439463	AE binding protein 1	AEBP1	-1.37
R41787	Hs.129340	Cadherin 13, H-cadherin (heart)	CDH13	3.94
W49619	Hs.334131	Cadherin 2, type 1, N-cadherin (neuronal)	CDH2	-3.06
AA598507	Hs.1640	Collagen, type VII, alpha 1	COL7A1	0.64
AA012944	Hs.8867	Cysteine-rich, angiogenic inducer, 61	CYR61	-1.45
AA777187	Hs.8867	Cysteine-rich, angiogenic inducer, 61	CYR61	-1.01
H44785	Hs.443518	Dystonin	DST	1.23
AA704323	Hs.8117	ErbB2 interacting protein	ERBB2IP	0.79
AA115902	Hs.2799	Hyaluronan and proteoglycan link protein 1	HAPLN1	1.99
AA663440	Hs.286229	Plexin C1	PLXNC1	-1.14
W86145	Hs.409546	Rho GTPase activating protein 5	ARHGAP5	0.65
AI364513	Hs.349656	Scavenger receptor class B, member 2	SCARB2	0.55
AA776891	Hs.349656	Scavenger receptor class B, member 2	SCARB2	0.60
AA449715	Hs.15154	Sushi-repeat-containing protein, X-linked	SRPX	-1.29
AA464532	Hs.164226	Thrombospondin 1	THBS1	-2.34
N58107	Hs.2257	Vitronectin (serum spreading factor, somatomedin B)	VTN	-1.52
Mitosis				
H90902	Hs.534403	CDC23 (cell division cycle 23, yeast, homolog)	CDC23	1.13
R20737	Hs.534403	CDC23 (cell division cycle 23, yeast, homolog)	CDC23	1.12
N35067	Hs.404501	Cell division cycle 2-like 5 (cholinesterase-related cell division controller)	CDC2L5	0.46
AI291082	Hs.404501	Cell division cycle 2-like 5 (cholinesterase-related cell division controller)	CDC2L5	0.55
N64731	Hs.386198	Echinoderm microtubule-associated protein like 4	EML4	-0.75
AA430032	Hs.350966	Pituitary tumour-transforming 1	PTTG1	1.07
AI362866	Hs.350966	Pituitary tumour-transforming 1	PTTG1	0.95
H73968	Hs.9329	TPX2, microtubule-associated protein homolog	TPX2	0.78
R80790	Hs.93002	Ubiquitin-conjugating enzyme E2C	UBE2C	1.55
AA430504	Hs.93002	Ubiquitin-conjugating enzyme E2C	UBE2C	1.53
AA599145	Hs.13512	ZW 10 homolog, centromere/kinetochore protein	ZW10	-1.62
Extracellular				
AA700054	Hs.3416	Adipose differentiation-related protein	ADFP	-2.00
AA701963	Hs.75313	Aldo-keto reductase family 1, member B1 (aldose reductase)	AKR1B1	-2.69
AA991590	Hs.268571	Apolipoprotein C-I	APOC1	-3.26
AA873159	Hs.268571	Apolipoprotein C-I	APOC1	-3.22
T70891	Hs.110675	Apolipoprotein E	APOE	-2.27
AA101875	Hs.434488	Chondroitin sulphate proteoglycan 2 (versican)	CSPG2	-0.84
AA012944	Hs.8867	Cysteine-rich, angiogenic inducer, 61	CYR61	-1.45
AA777187	Hs.8867	Cysteine-rich, angiogenic inducer, 61	CYR61	-1.01
AI382562	Hs.121150	Taxilin	DKF Z p451J0118	-0.65
H44785	Hs.443518	Dystonin	DST	1.23
N79484	Hs.81071	Extracellular matrix protein 1	ECM1	-0.67
H17615	Hs.11494	Fibulin 5	FBLN5	-2.56
AA417274	Hs.433827	Follistatin-like 3 (secreted glycoprotein)	FSTL3	-0.94
W81526	Hs.113882	Gamma-aminobutyric acid (GABA) A receptor, delta	GABRD	0.66
AA115902	Hs.2799	Hyaluronan and proteoglycan link protein 1	HAPLN1	1.99
N74623	Hs.251664	IGF2 (somatomedin A)	IGF2	8.04
N54596	Hs.251664	IGF2 (somatomedin A)	IGF2	6.58
N54596	Hs.251664	IGF2 (somatomedin A)	IGF2	6.47
AA598601	Hs.450230	IGF binding protein 3	IGFBP3	2.15
AA447781	Hs.406475	Lumican	LUM	-1.75
AA071473	Hs.153647	Matrilin 2	MATN2	-1.41
H22922	Hs.371768	Manic fringe homolog (<i>Drosophila</i>)	MFNG	-1.09
R72075	Hs.172816	Neuregulin 1	NRG1	-0.63
AA086038	Hs.410477	Phospholipase A2 receptor 1.180 kDa	PLA2R1	-2.17
R38933	Hs.274404	Plasminogen activator, tissue	PLAT	-0.78
AA485893	Hs.78224	Ribonuclease, RNase A family, 1 (pancreatic)	RNASE1	-2.26
R45235	Hs.118684	Stromal cell-derived factor 2	SDF 2	-0.57
R33439	Hs.24640	Semaphorin/A	SEMA7A	1.20
R51912	Hs.12409	Somatostatin	SST	0.93

Table 2 Continued

Accession	UG cluster	Gene name	Symbol	Log ratio
AA085319	Hs.25590	Stanniocalcin 1	STC1	0.66
AA464532	Hs.164226	Thrombospondin 1	THBS1	-2.34
AA099153	Hs.245188	Tissue inhibitor of metalloproteinase 3	TIMP3	-1.78
AA479202	Hs.245188	Tissue inhibitor of metalloproteinase 3	TIMP3	-2.12
N58107	Hs.2257	Vitronectin (serum spreading factor, somatomedin B)	VTN	-1.52

adrenocortical cancer, was the most up-regulated transcript. *FGFR1* and *FGFR4* were up-regulated growth factor receptors.

Enrichment analysis and GO classification of differentially expressed genes in cancers

An enrichment analysis of the chromosomal location of differentially expressed genes revealed that overexpression on chromosome 5 and underexpression on chromosome 2 and 1 were significant. To investigate which biological processes the differentially expressed genes were involved in, GO annotations were used. Enrichment analysis of the highest ranked differentially expressed transcripts according to statistical significance comparing carcinomas to all other samples showed that anti-apoptosis and cell cycle, were over-represented among genes with altered transcript levels (Supplementary file 1C). A subset of genes with GO annotation adhesion, mitosis or extracellular location is presented in Table 2 since these categories are of interest in the context of cancer development.

Verification by qRT-PCR

To confirm microarray results, four of the most significantly differentially expressed genes were analysed with qRT-PCR. qRT-PCR was performed for *ALDH1A1*, *IGF2*, *USP4* and *UFD1L*. This confirmed *IGF2* as significantly overexpressed and *ALDH1A1* as significantly underexpressed in carcinomas (with the exception of sample 2931) compared to all other samples (Fig. 3). The differences were too small and the variation was too high for *USP4* and *UFD1L* to generate a significant difference. On average the up-regulation was 1.3- and 1.4-fold, respectively, in cancer versus non-cancer samples. The corresponding values for *IGF2* was 144-fold up-regulation and 12-fold down-regulation of *ALDH1A1*. Ratios were calculated in a similar way for the array data by taking the average of the ratio for those genes in the cancer samples versus the reference RNA and then divide that with the corresponding ratio for the non-cancer samples. The corresponding fold over-expression for *USP4* and *UFD1L* was 44 and 32 respectively. The corresponding values for *IGF2* was 388-fold up-regulation and for *ALDH1A1* 11-fold down-regulation respectively.

USP4 protein expression is increased in adrenocortical carcinomas

The microarray data described here indicate a significant overexpression of the deubiquitinating enzyme USP4 (also known as UNP for ubiquitous nuclear protein) in adrenocortical carcinomas compared to adenomas. To test if the increased transcription correlates with elevated protein levels we prepared lysates from tumour biopsies and performed western blot analysis using an anti-USP4 antibody (Fig. 4). A clear increase in a couple of close migrating bands corresponding to the expected

Figure 3 Verification by qRT-PCR. Relative expression levels of (A) *IGF2* and (B) *ALDH1A1*. The array data are displayed as filled squares on the left axes as log 2 ratios versus the common reference RNA and the real time PCR data as open squares on the right axes as ratios in relation to 18S rRNA.

Figure 4 Usp4 protein levels are increased in adrenocortical carcinomas compared with adenomas. Western blot analysis using an anti-USP4 antibody showing increased expression of USP4 protein in adrenocortical carcinomas as compared to adenomas. Underlined are paired samples of tumour (T) and normal adrenal cortex (N) from the same patient. An additional smaller product reacting with the USP4 antibody shows increased expression in carcinomas (asterisk).

size of endogenous USP4 was detected in the carcinoma samples as compared to normal reference. Smaller bands (~ 75 kDa) and additional weak bands reacting with the antibody may indicate potential splice variants or cleavage products of USP4.

Survival analysis and gene expression in cancer tumours

In order to examine if the survival of cancer patients were reflected in the gene expression patterns, cancer data were subject to hierarchical clustering using the entire filtered probe set where each gene profile had been normalised to the same range. The resulting two subclusters had different outcomes; one cluster contained patients with short survival (< 9 months) and the other patients with long survival (> 67 months) suggesting that gene expression profiles may be used for prediction of survival. In Fig. 5A, the dendrogram based on the entire probe set is displayed together with the gene expression profiles of 30 up- and 30 down-regulated genes. The Kaplan–Meier survival curves of the two subclusters are shown in Fig. 5B.

Differential expression associated with hormonal activity of benign tumours

The benign tumour sample set consisted of five Cushing, four aldosteronomas and eight non-hormonal over-producing incidentalomas. Comparing each subtype to the other samples we could detect 11 genes with expression levels connected to aldosteronomas using the statistical criteria for differential expression described in Materials and methods (Table 3). In Supplementary file 2A, which can be viewed online at <http://www.eje-online.org/supplemental/>, data for all probes is shown. Differentially expressed genes were not identified for the other subtypes. Among the dysregulated genes were, vascular endothelial growth factor B (VEGFB) and oxysterol binding protein (OSBP). Comparison of aldosteronomas versus incidentalomas revealed several significantly dysregulated genes as detailed in Supplementary file 2B. Mevalonate kinase (MVK) was up-regulated in aldosteronomas as well as

peroxiredoxin 5 (PRDX5), while tumour necrosis factor receptor super family 25 (TNFRSF25) was down-regulated (Supplementary files 2B and C).

Discussion

It is important to elucidate the relation and differences between benign and malignant adrenocortical tumours, both in terms of obtaining an increased understanding for the underlying molecular mechanisms as well as to enhance possibilities for correct diagnosis and prognosis for the individual patient. Previous studies have demonstrated that adrenocortical carcinomas display specific and more numerous chromosomal and/or genetic alterations than adenomas (4, 5), which could suggest a characteristic malignant genotype. In a previous study we showed that malignant gene expression profiles are distinguishable from those of benign tumours (16). In this extended study we also included normal adrenal cortex to ascertain that the characteristics that separated cancers from benign tumours were associated with malignancy and not present in normal adrenocortex. We found potentially useful expression profiles which could enable differentiation between carcinomas and non-malignant adrenocortical tissue (normal adrenocortex and adenomas), and furthermore between different subclasses of adenomas. We could also show that in the malignant group tumours from patients with long survival and tumours from patients with short survival fell into two separate cluster groups.

The IGF signalling pathway plays a major role in several cancer forms and is interesting considering development of therapeutics and as a diagnostic approach. IGF2 was up-regulated in adrenocortical carcinomas in this study as in previous published gene expression profiling studies on adrenocortical tumours (21–25). Elevated expression levels of IGF2 has been detected in several other cancer forms (26–28). In a study of ovarian cancer, expression levels of IGF2 have been demonstrated to be inversely correlated to survival (29). Serum levels of IGF2 have been proven to have biomarker potential for tumour staging of prostate cancer as a complement to PSA (30). IGF2 is thus a

relatively general cancer marker but still has the potential to become very useful to distinguish adrenocortical adenomas from carcinomas.

Several of the up-regulated genes in carcinomas, such as the deubiquitinating enzymes *USP4* and *USP38*, the ubiquitin conjugating enzyme *UBE2C*, the small ubiquitin-like modifier *SUMO-1* and *UFD1L* are involved in the ubiquitination pathway (Fig. 2). Post-translational modification by ubiquitin has emerged as a major regulatory event that is essential for cellular processes such as cell cycle progression, signal transduction, transcriptional regulation, and apoptosis (31). *USP4*, which was among the most up-regulated genes is an enzyme of yet unclear function, however, its deregulation has previously been linked to cancer and *USP4* has been ascribed oncogenic (32, 33) as well as having tumour suppressor functions (34) indicating that it may be a multifunctional protein. *USP4* has previously been reported to interact with the retinoblastoma tumour suppressor, pRb, the pocket proteins, p107 and p130 (35, 36), the ubiquitin ligase Ro52 (37) and the transmembrane G-coupled adenosine 2A receptor for which it has been suggested to function to relax the endoplasmic reticulum quality control mechanisms, resulting in increased cell surface expression of the receptor (38). The role of *USP4* in adrenocortical carcinomas is however, still unexplored. *UBE2C* (also known as *UbcH10*) is a ubiquitin conjugating enzyme involved in cell cycle regulation (39) and its over expression has been suggested to be a valid indicator of human cancers of various tissue origin (40–42).

Differentially expressed genes in carcinomas showed overrepresentation for location on chromosome 5 among genes up-regulated in cancer, while down-regulated genes were enriched for chromosome 1 and 2. These observations overlap with those made with CGH showing gains on chromosome 5 and losses on chromosome 2 in adrenocortical cancers (4).

As previously mentioned, we found an interesting cancer case (ID 2931), that surprisingly clustered with normal cortex and adenomas in the unsupervised hierarchical clustering (Fig. 1). This patient had no metastasis at the time of surgery but received mitotane as adjuvant chemotherapy. The patient is still alive 67 months after surgery without any evidence of residual disease or metastasis. The tumour showed low proliferation with MIB-1/Ki-67, <3% of activity. Interestingly, this case also showed gene expression levels similar to the 'normal or adenoma' pattern in the qRT-PCR for *IGF2* and *ALDH1A1* (Fig. 3).

Figure 5 (A) Hierarchical clustering of cancers based on the entire filtered probe set results in two subclusters; cluster 1 and cluster 2. A subset of 30 plus 30 genes with differential expression are displayed beneath. (B) Kaplan–Meier survival curve displays longer survival times for cluster 1 cases as compared to cluster 2 cases.

Table 3 Differentially expressed genes comparing aldosteronomas to all other tissue samples, (cut off: log fold change > 1, adjusted *P* value < 0.001).

Accession	UniGene	Gene name	Symbol	Log fold change	Adj. <i>P</i> value	<i>B</i>
R99527	Hs.8162	Hypothetical protein MGC39372	MGC39372	2.17	5.09×10^{-5}	10.16
AA398335	Hs.10414	Hypothetical protein FLJ10748	FLJ10748	2.26	5.09×10^{-5}	9.56
AA441933	Hs.79386	Leiomodin 1 (smooth muscle)	LMOD1	2.33	5.09×10^{-5}	9.54
AA630120	Hs.78781	Vascular endothelial growth factor B	VEGFB	1.24	2.93×10^{-4}	7.59
R07846	Data not found			3.71	2.93×10^{-4}	7.49
W92795	Hs.434386	Hypothetical protein LOC201229	LOC201229	1.55	4.01×10^{-4}	7.03
AA454564	Hs.323396	Hypothetical protein RP1-317E23	LOC56181	1.25	5.17×10^{-4}	6.65
AA775249	Hs.6527	G protein-coupled receptor 56	GPR56	-1.63	6.42×10^{-4}	6.33
AA012822	Hs.24734	Oxysterol binding protein	OSBP	1.35	7.07×10^{-4}	6.14
R45592	Hs.153610	Regulating synaptic membrane exocytosis 2	RIMS2	2.51	7.11×10^{-4}	6.04
AA282936	Hs.240	M-phase phosphoprotein 1	MPHOSPH1	-1.40	8.87×10^{-4}	5.74

Two recent studies have addressed the connection between gene expression levels and prediction of survival (23, 24). Giordano *et al.* could show that the cancer gene expression signatures divided into two subclusters with different survival outcome suggesting microarrays as a prognostic tool. De Reyniès *et al.* also found two subclusters with different survival outcomes. In addition, two specific genes (*PINK1* and *BUB1B*) were selected as biomarkers, which in combination could be used for prediction of survival. In our study two subclusters with different survival outcomes could be observed as well. Due to the limited sample set size, we chose not to select any specific markers based on these. However, we could confirm that *PINK1* and *BUB1B* also could be used as predictors for survival in our cases (data not shown) although *BUB3* was a better predictor than *BUB1B*. A weakness of the analysis in our study to take into consideration is that all diseased patients but one (372) are females.

Regarding tumours with different hormonal activity, differences in expression patterns of genes related to steroid hormone synthesis are of special interest. Several interesting genes involved in intermediate metabolism were present on the array, such as the oxysterol binding protein (*OSBP*) which was found to be up-regulated in aldosteronomas. This gene is involved in sterol transport from lysosomes to the nucleus, down-regulating the expression of other genes such as *LDL-receptor*, *HMG-CoA reductase* and *HMG synthetase*, involved in lipid synthesis. An early enzyme in steroid synthesis is mevalonate kinase (*MVK*), which was up-regulated in aldosteronomas compared to incidentalomas. Besides genes directly involved in hormonal metabolism, many other genes were found to be differentially expressed in aldosteronomas, among these *VEGFB*, adenosine deaminase (*ADA*), inositol 1,3,4-triphosphate 5/6 kinase (*ITPK1*), ubiquitin thiolesterase 1 (*UCHL1*), tumour necrosis factor receptor super family member 25 (*TNFRSF25*), and the V-abl Abelson murine leukaemia viral oncogene homolog 1 (*ABL1*; Supplementary file 2A and B).

Conclusions

Through the transcriptional profiling, different subtypes of functional adenomas and carcinomas may be distinguished based on their specific molecular signature. This study confirms and extends the findings of previous studies in this respect. Our utilisation of a whole genome cDNA microarray may serve as a complement to gene expression data retrieved with Affymetrix high density microarrays and cDNA macroarrays. Further investigation of the relevance of genes with subtype specific differential expression has the potential to eventually lead to clinical breakthroughs together with the findings of the larger studies recently published. Genes with expression levels connected to malignancy, survival and hormonal activity were detected in our data. Identification of these differentially expressed genes may enhance our understanding of the molecular biology of adrenocortical tumour development and its functional phenotype, and aid in creating new diagnostic and prognostic tools.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. C Larsson holds a patent share for *VEGFB*.

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