

DIFFERENTIAL RNA EXPRESSION IN BENIGN AND MALIGNANT ADRENOCORTICAL TUMOURS

David Velázquez-Fernández



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DIFFERENTIAL RNA EXPRESSION IN BENIGN AND MALIGNANT ADRENOCORTICAL TUMOURS

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Per ardua ad astra, et ex astris, Scientia
"Through adversity to the stars, and from the stars, Knowledge"

Bene diagnoscitur, bene curatur
"Something that is well diagnosed can be cured well"

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1 ABSTRACT

Adrenocortical cancer is a rare but very aggressive endocrine tumor, with a yearly incidence of 0.5 to 2 cases per million inhabitants, accounting for 0.2% of all cancer related deaths. This contrasts to the high prevalence of adrenocortical tumors up to 9%, which are detected with the increasing use of modern imaging methods such as ultrasound, computed tomography and magnetic resonance imaging. The most frequent clinical presentation is an abdominal mass without any other specific symptom. Although there are some radiological and histological features indicative of adrenocortical cancer, the diagnosis is difficult. Markers for the distinction of malignant tumors are needed. Genomic profiling tools such as cDNA microarrays have revealed transcriptional signatures, enabling to predict malignant behavior in several different neoplasms. This technique can also provide a valuable insight into the molecular mechanisms involved in cancer development and progression, to help identifying genes of potential therapeutic and diagnostic importance.

The aim of the present study was to investigate the differences in the transcriptional profiles between benign and malignant adrenocortical tumors through the use of microarray technology, to further characterize these differences between hormonally producing adrenocortical tumors, incidentalomas and normal adrenocortical samples, and to find genes that can serve as potential markers for the diagnosis of adrenocortical cancer.

In paper I seven patients with adrenocortical carcinomas and thirteen with adenomas were studied. Total RNA was obtained from all clinical samples. Gene expression analysis of these tumors was conducted by cDNA microarray. Transcriptional profiles were homogeneous among adenomas and heterogeneous in carcinomas. Hierarchical clustering and self-organizing maps could clearly separate carcinomas from adenomas. Among genes that were upregulated in carcinomas were two ubiquitin related genes (USP4 and UFD1L) and several insulin-like growth factor related genes (IGF2, IGF2R, IGFBP3 and IGFBP6). Among other genes that were downregulated in carcinomas were several genes related to cell metabolism (ALDH1A1, RARRES2, CYBRD1 and GSTA4), a cytokine (CXCL 10) and a cadherin 2 (CDH2). Through the use of cDNA arrays adrenocortical adenomas and carcinomas may be clearly distinguished, based on their specific molecular signature.

In paper II we analyzed mRNA expression profiles in adrenocortical samples: nine hormonally active benign tumors (five cortisol and four aldosterone producing tumors), eight hormonally inactive benign tumors (incidentalomas) eight adrenocortical carcinomas and four normal adrenocortical tissue samples. Through the use of cDNA arrays, adenomas and carcinomas may be clearly distinguished based on their specific molecular signature. Several genes were identified which were expressed in different benign tumor subtypes. Identification of these differentially expressed genes may enhance our understanding of the molecular biology of adrenocortical tumor development and its functional phenotype, and may create possibilities for new diagnostic, prognostic and therapeutic tools.

2 LIST OF ARTICLES

I. **Expression Profiling of Adrenocortical Tumors Suggests a Molecular Signature of Malignancy.** *Velázquez-Fernández D, and Laurell C, Geli J, Höög A, Odeberg J, Kjellman M, Lundeberg J, Hamberger B, Nilsson P and Bäckdahl M. Surgery (ahead in printing).*

II. **Gene Expression Profiles allow the Molecular Classification of Adrenocortical Lesions.** *Laurell C and Velázquez-Fernández D, Enberg U, Juhlin C, Geli J, Höög A, Odeberg J, Kjellman M, Lundeberg J, Larsson C, Hamberger B, Bäckdahl M and Nilsson P.*

* The papers are referred to by their roman numbers.

3 LIST OF ABBREVIATIONS

CT	Computed Tomography
MRI	Magnetic Resonance Imaging
US	Ultrasonography
CGH	Comparative Genomic Hybridization
RNA	Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
DNA	Deoxyribonucleic acid
cDNA	Complementary Deoxyribonucleic acid
dNTP	Deoxynucleotidetriphosphate
PCR	Polymerase Chain Reaction
RT-PCR	Real Time PCR
UHRR	Universal Human Reference RNA
LLID	Locus Link Identification
NCBI	National Center for Biotechnology Information
GO	Gene Ontology
DMSO	Dimethyl Sulphoxide
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
MEN	Multiple Endocrine Neoplasia
MC2R	Melanocortin 2 Receptor
DOC	Deoxycorticosterone
IGF2	Insulin-like Growth Factor 2
IGF2R	Insulin-like Growth Factor 2 Receptor
ALDH1A1	Aldehyde Dehydrogenase 1 family, member A1
USP4	Ubiquitin Specific Protease 4
HSD3B1	3 β -Hydroxysteroid dehydrogenase/ Δ -5 isomerase type 1
UFD1L	Ubiquitin Fusion Degradation 1-like
IGFBP3	Insulin-like Growth Factor Binding Protein 3
IGFBP6	Insulin-like Growth Factor Binding Protein 6
FGFR1	fibroblast growth factor receptor 1
FGFR4	fibroblast growth factor receptor 4
MET	met proto-oncogene (hepatocyte growth factor receptor)
VEGFB	vascular endothelial growth factor B
CXCL10	Chemokine (C-X-C motif) Ligand 10
RARRES2	Retinoic Acid Receptor Responder (tazarotene induced) 2
CYBRD1	Cytochrome b reductase 1
GSTA4	Glutathione S-transferase A4
CDH2	Cadherin 2, type 1, N-cadherin (neuronal)
OSBP	Oxysterol Binding Protein
MVK	Mevalonate Kinase
FADS2	Fatty Acid Desaturase 2
ADA	Adenosine Deaminase
ITPK1	Inositol 1,3,4-triphosphate 5/6 kinase
UCHL1	ubiquitin thiolesterase 1
TNFR SF25	Tumour Necrosis Factor Receptor Superfamily member 25
ABL1	V-abl Abelson murine leukemia viral oncogene homolog 1

4 BACKGROUND

An adrenal neoplasm may arise from either the adrenal cortex or medulla. These layers come from different embryological origins, the mesoderm and the ectoderm, respectively with different endocrine functions (Rosai and Ackerman 2004, Barwick et al 2005, Lin et al 2005). The adrenal cortex secretes mineralocorticoids, glucocorticoids and sex hormones, mainly in coordination with the hypothalamus-pituitary axis. The adrenal medulla secretes the catecholamines adrenaline and noradrenaline (Boushey et al 2001, Rosai and Ackerman 2004, Lin et al 2005). Neoplasms arising from the cortex are called adrenocortical tumors and can be benign or malignant (Rosai and Ackerman 2004).

Epidemiology

Adrenocortical carcinoma is a relatively rare but very aggressive endocrine tumor, with a yearly incidence of up to 2 cases per million inhabitants, accounting for 0.2% of cancer related deaths (Kjellman et al 2001, Lin et al 2005). Mean survival time for adrenocortical cancer is less than 12 months for unresectable tumors. For resectable tumors the overall 5 year survival is less than 40% (Dackiw et al 2001, Vassilopoulou-Sellin et al 2001). Adrenocortical tumors are nowadays discovered earlier and more often due to newer and more sophisticated radiological methods. When an adrenal mass is found incidentally by radiological examination such as computed tomography, it is commonly referred as an incidentaloma (Lin et al 2005). In autopsy studies, the prevalence of adrenocortical tumors in the population is as high as 9% (Hedeland et al 1968, Boushey et al 2001, Sidhu et al 2003).

Etiology

Adrenocortical tumors may have sporadic or familial origin (Koch et al 2002, Libé et al 2005). The most frequent is the sporadic origin, which accounts for more than 90% of the cases. Some epidemiological studies have suggested an increased risk of adrenocortical tumors with the use of oral contraceptives and smoking (Dackiw et al 2001, Allolio et al 2004). However, etiology is still unknown in sporadic adrenocortical tumors. There are some familial syndromes including adrenocortical tumors, such as Hereditary Adrenocortical Carcinoma, Multiple Endocrine Neoplasia type 1 (Marx et al 2005), Li-Fraumeni Syndrome and Beckwith-Wiedemann Syndrome (Dackiw et al

2001, Sidhu et al 2003, Allolio 2004). Li-Fraumeni Syndrome is characterized by the development of several malignancies such as breast cancer, sarcomas and adrenocortical cancer. A germline mutation of the p53 tumor suppressor gene located at 17p13 locus can be found in about 70% of affected families. In sporadic cases of adrenocortical cancer, the accumulation of an abnormal p53 protein has been correlated with a more aggressive clinical course (Kirschner et al 2002, Sidhu et al 2003, Alloilio et al 2004, Pianovski et al 2005). Beckwith-Wiedemann Syndrome is associated with malignancies such as Wilm's tumor, hepatoblastoma and adrenocortical cancer (Williams et al 2005). This syndrome has been mapped to the 11p15.5 locus, which includes the IGF2, H19 and p57/Kip2 genes. Paternal isodisomy has been related to the IGF2 overexpression in cases with Beckwith-Wiedemann Syndrome (Kirschner et al 2002). It has been demonstrated that some sporadic cases of adrenocortical cancer have a rearrangement at the 11p15 locus, causing an overexpression of IGF2 or loss of H19, which suppresses IGF2 expression (Kjellman et al 2001, Sidhu et al 2003, Allolio et al 2004). An increased expression level of IGF2 has been reported in sporadic adrenocortical cancer using microarray technique (Giordano et al 2003).

Diagnosis

Adrenocortical tumors may be diagnosed based on their hormonal production, clinical picture and radiological appearance (Vassilopoulou-Sellin et al 2001, Sidhu et al 2003, Lin et al 2005). These tumors can secrete abnormally high levels of adrenocortical hormones such as glucocorticoids, mineralocorticoids or less frequent, sex hormones. Based on the hormonal pattern, particular phenotypic features appear (Boushey et al 2001, Sidhu et al 2003). A common syndrome associated with adrenocortical tumors in adults is adrenal Cushing's syndrome. The adrenocortical tumor produces glucocorticoids, mainly cortisol, which is associated with a characteristic clinical picture (Lin et al 2005).

Adrenocortical cancers are functional in approximately 60% and produce not only cortisol but often considerable amounts of androgens and steroid precursors (Ng et al 2003, Sidhu et al 2003, Michalkiewicz et al 2004). Therefore, signs of virilization may appear in women. The combination of adrenal Cushing's syndrome and virilization is evident in 20 to 30% of functional adrenocortical cancer (Ng et al 2003, Alloilio et al 2004, Michalkiewicz et al 2004, Lin et al 2005). Patients with nonfunctioning adrenocortical cancer usually present with vague symptoms such as abdominal fullness,

pain, nausea, indigestion, general malaise, weight loss and weakness (Dackiw et al 2001). Aldosterone producing tumors have some characteristic clinical features, basically hypertension and hypokalemia. A careful biochemical evaluation should be performed in these cases (Mussig et al 2005). Frequently these patients are candidates for surgery. Preoperative localization studies play an important role for the appropriate surgical decision.

A primary clinical approach for adrenocortical tumors is based on hormonal screening (Boushey et al 2001, Lin et al 2005, Reynolds et al 2005). Unfortunately, hormone concentrations by themselves are usually of limited help in predicting malignancy. A complete biochemical evaluation is recommendable and may serve as marker of disease progression (Sidhu et al 2003, Lin et al 2005). This includes serum electrolytes, blood glucose, liver function tests, careful history, physical examination and radiological assessment. Hormonal secretion pattern provides an important diagnostic tool for the assessment of adrenocortical tumors. For example elevation of the dehydroepiandrosterone sulphate (DHEAS) may indicate malignancy (Ng et al 2003, Sidhu et al 2003, Allolio 2004). Gene mutations of adrenocortical cells may lead to enzymatic deficiencies in the steroid metabolic pathway with abnormal steroid intermediates or metabolites in the urine and plasma (Grondal et al 1990 and 1991). Some of these steroids seem to be more common in patients with adrenocortical cancer than in benign adrenocortical diseases and healthy subjects, such as the increased secretion of 11-deoxytetrahydrocortisol and 3 β -hydroxy-5-enosteroids. Excretion of 11-deoxytetrahydrocortisol and metabolites of cortisol precursors have been found in both functional and non functional adrenocortical cancer.

Imaging is one of the most useful clinical tools for evaluating adrenocortical tumors. Characteristics such as size, density, architecture and vascularity, are important for assessing the lesion's nature (Boushey et al 2001, Sidhu et al 2003, Thorin-Savouré et al 2005). Based on computed tomography (CT) images, surgeons nowadays can decide if open or laparoscopic adrenalectomy is suitable. Magnetic resonance imaging (MRI) is also a useful imaging method for discerning adrenocortical cancer and particularly sensitive for identifying metastatic disease. MRI criteria for adrenocortical cancer diagnosis include lack of fat suppression, heterogeneous T2 signalling, gadolinium enhancement and slow wash-out (Bornstein et al 1999, Dackiw et al 2001). Early reports on very sensitive detection of adrenocortical cancer using ^{11}C -metomidate and

fluorodeoxyglucose as tracers for positron emission tomography (PET) have shown promising results (Khan et al 2002, Rodriguez-Galindo et al 2005, Eriksson et al 2005).

Some histopathological features can be useful for discerning adenomas from carcinomas (Rosai and Ackerman 2004). Adrenocortical adenomas are characteristically small, with a homogeneous appearance, well encapsulated, rarely exceeding 5 cm as their greatest diameter or 20-50 g in weight. In contrast, most cancers are larger, weigh more (more than 100 g), and frequently have necrosis, hemorrhage as well as vascular and/or capsular invasion (Rosai and Ackerman 2004, Rodriguez-Galindo et al 2005). In a classical paper, Weiss et al (Medeiros et al 1992, Rosai and Ackerman 2004) listed nine morphologic criteria which can be useful for discerning cancer from adenomas:

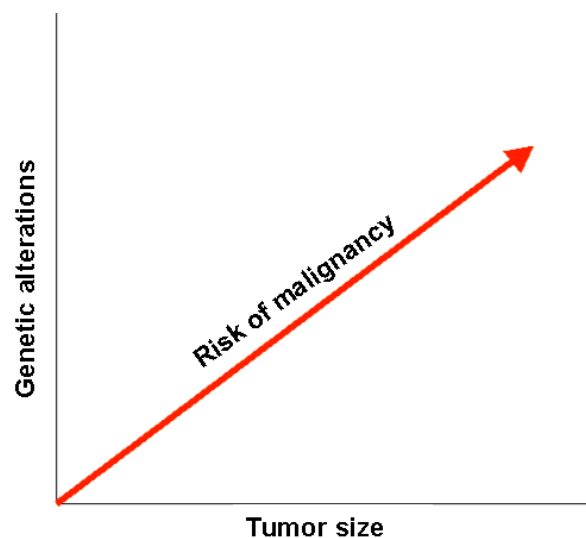
- High nuclear grade (III or IV)
- Mitotic rate > 5 per 50 high-power fields
- Atypical mitotic figures
- Eosinophilic tumor cell cytoplasm ($\geq 75\%$ of tumor cells)
- Diffuse architecture ($\geq 33\%$ of tumor)
- Necrosis
- Venous invasion (smooth muscle in wall)
- Sinusoidal invasion (no smooth muscle in wall)
- Capsular invasion

Three or more of these criteria found with histopathologic examination are indicative of an adrenocortical tumor with malignant potential.

Immunohistochemistry can add information for discerning adenomas from cancer. The expression of keratins is higher in adenomas than in cancer. In contrast, the expression of vimentin is lower in adenomas than in cancer (Rosai and Ackerman 2004). Other proteins have also been proposed as useful markers for cancer such as: agNOR, neuron-specific enolase, chromogranin A, S-100 protein, Leu-7, Ki-67 (MiB-1), epithelial membrane antigen, DNA topoisomerase II alpha, CREB, KL1, AE1, AE3, 3- β -hydroxysteroid dehydrogenase, adrenal-4-binding protein, p53 and telomerase activity (Wachenfeld et al 2001, Rosenberg et al 2003, Stratakis et al 2003). Unfortunately none of these markers is able to completely classify these tumors.

Size is the single best indicator for malignancy in adrenocortical tumors (Kjellman et al 1996, Dackiw et al 2001). It has been demonstrated that size has a positive correlation

with number of genetic alterations. Thus, small lesions (<3 cms) are usually benign and harbor few genetic alterations, while bigger lesions (>6 cms) are frequently malignant and have more genetic alterations. It has been found that only 2.5% of adrenocortical cancers are less than 4 cm in greatest diameter. Therefore, size is helpful for surgical decision. Surgical excision is usually indicated when the tumor diameter is above 3-4 cm (Boushey et al 2001, Sidhu et al 2003, Rosai and Ackerman 2004). However, this limit varies throughout centers in the world.



There is no single clinical, morphological or biochemical marker available to conclusively distinguish an adenoma from cancer. Only metastasis or local invasion can establish the ultimate diagnosis of adrenocortical cancer, which in many cases is not apparent until the follow up period (Rosai and Ackerman 2004).

Treatment

Complete surgical resection is the standard treatment for adrenocortical tumors and is the best predictor of long-term survival (Dackiw et al 2001, Icard et al 2001, Kotzampassakis et al 2005, Shen et al 2005). Surgery is also the treatment of choice for patients with secondary tumors and for recurrent disease. A complete surgical resection

is the only potentially curative treatment (Boushey et al 2001, Sidhu et al 2003). There are two surgical approaches for adrenocortical tumors. Open surgery is indicated whenever cancer is suspected and also in large tumors (>8 cm in their greatest diameter) while otherwise minimal access surgery is the preferred approach for adrenocortical tumors because of its shorter hospital stay, better cosmesis and less postoperative pain (Ramacciato et al 2005, Walz et al 2005, Diner et al 2005, Ishidoya et al 2005, Rubinstein et al 2005). Radical resection should be performed in all cases with a high suspicion of malignancy. To avoid tumor spillage, the tumor capsule must remain intact. Invasion or adherence of carcinoma to adjacent organs often requires en bloc resection of the kidney, spleen, partial hepatectomy or pancreatectomy (Ng et al 2003, Viterbo et al 2005, Kumar et al 2005). In addition, lymphadenectomy should be considered. The presence of a tumor thrombus in the renal vein or inferior cava does not preclude a radical and complete resection. When radical surgery is not possible, palliative surgery and debulking to reduce hormonally active tumor tissue, often gives symptomatic relief but no impact on patient's survival. Surgical resection of recurrent disease is also an important therapeutic option associated with longer survival, although cure is seldom achieved (Dackiw et al 2001, Sidhu et al 2003).

Chemotherapeutic drugs such as mitotane has demonstrated efficacy for adrenocortical cancer (Vassilopoulou-Sellin et al 2001, Alloilio et al 2004). Clinical response rate to mitotane is variable across institutions but in general it is less than 35%. This response is mostly partial and transient with only occasional complete remission. The role of mitotane as adjuvant therapy is currently a matter of debate (Dackiw et al 2001, Icard et al 2001, Lin et al 2005, Hahner et al 2005). However, this drug remains the first alternative for treating adrenocortical cancer (Ahlman et al 2001, Boushey et al 2001, Vassilopoulou-Sellin et al 2001, Ng et al 2003, Rodriguez-Galindo et al 2005).

Other cytotoxic agents such as streptozocin and etoposide have been used alone or in combination with mitotane. Results are variable in different published studies but none have demonstrated satisfactory results (Boushey et al 2001, Dackiw et al 2001, Khan et al 2004). The highest response rate (53.5%) has been observed in a phase II multicentre trial using the combination of etoposide, doxorubicin and cisplatin given together with continuous mitotane. The combination of mitotane and streptozocin has also been demonstrated to increase survival compared to patients without treatment after complete surgical resection (Khan et al 2000, Alloilio 2004). Radiotherapy has also a limited role

in adrenocortical cancer, mainly recommended for palliating pain due to bone metastases and occasionally with a limited value as adjuvant treatment (Dackiw et al 2001).

Genetics of adrenocortical tumors

Cancer is the end result of clonal expansion of a specific cell population, which have acquired a number of non-lethal genetic alterations favoring tumor phenotype and development. Numerous specific alterations may be detected in cancer cells, affecting from entire genomes, chromosome number or structure and chromatin assembly or specific genes and sequences. These changes can be detected by several methods and may serve to diagnose cancer (Kjellman et al 2001, Koch et al 2002, Sidhu et al 2003). One of these methods is DNA ploidy analysis which has been shown to be a valuable tool for discerning cancer from benign adrenocortical tumors in some studies (Pignatelli et al 1998, Haak et al 1993, Stratakis et al 2003). Diploid DNA content is mainly demonstrated in normal or benign adrenal samples, while an aneuploid content is indicative for malignancy. Loss of heterozygosity analysis is a technique that is used to identify tumor suppressor genes. Loss of heterozygosity in loci 11p, 13q and 17p is often demonstrated in adrenocortical cancer (Zhao et al 1999, Bornstein et al 1999, Leboulleux et al 2001, Wachenfeld et al 2001, Gicquel et al 2001, Sidhu et al 2002, Bertherat et al 2003, Stratakis et al 2003, Sidhu et al 2003). Comparative genomic hybridization (CGH) is a molecular cytogenetic technique which allows the detection of DNA sequence copy number alterations across the entire genome in a single experiment (Dohna et al 2000, Bernard et al 2003). Regions with increased copy number reveal chromosomal sites that may contain oncogenes, whereas regions with decreased copy number may harbour tumor suppressor genes. Using CGH technique in adrenocortical tumors (Kjellman et al 1996 and 1999, Sidhu et al 2003), few genetic alterations has been demonstrated in small adenomas while larger adenomas and carcinomas showed an increasing number of chromosomal aberrations (Kjellman et al 1996 and 1999, Dohna et al 2000). The most common genetic aberrations found in these studies, were gains on chromosomal regions 4q (50%), 5p (50%) and 5q (50%), as well as losses on chromosomal regions in chromosomes 2 (50%), 11q (50%) and 17p (50%). Theoretically these regions could harbor critical loci or genes involved in the adrenocortical carcinogenesis (James et al 1999, Kjellman et al 2001, Bernard et al 2003).

In the last decade a new molecular biology tool microarray technology has been developed for revealing transcriptional profiles (Zhao et al 2002, Tay et al 2003, Chevillard et al 2004, Bassett et al 2005). This technique has provided the opportunity to assess the genetic expression (mRNA) of many thousands genes in one single experiment. With microarray technology it is possible to elucidate tumor classification, biomarkers for cancer and to predict therapeutic and prognostic markers (Pittman et al 2004, Finley et al 2004, Hannemann et al 2005, Roepman et al 2005).

Two studies have been published where this technology is used in adrenocortical tumors. In one study (Giordano et al 2003) the transcriptional profiles of 11 adrenocortical carcinomas, 4 adenomas, 3 normal and 1 macronodular hyperplasia were obtained. This group used an array exploring 10 500 unique genes. Ninety-one genes were upregulated in adrenocortical cancer compared to adenomas and normal tissue. IGF2, osteopontin and one serine threonine kinase, were the most upregulated genes in cancer. IGF2 was upregulated in 10 of 11 cancers (90%). During the preparation of this work, another microarray study was published (de Fraipont et al 2005) where 33 adrenocortical adenomas and 24 carcinomas were analyzed. Two hundred thirty genes were analyzed (187 cancer related genes, 34 adrenal cortex-specific genes and 9 control genes). IGF2 was highly upregulated in 75% of adrenocortical carcinomas. One downregulated gene called 3 β -Hydroxysteroid dehydrogenase/ Δ -5 isomerase type 1 (HSD3B1) was also found in carcinomas. HSD3B1 showed a low expression level in 81% of adrenocortical carcinomas and a high expression level in 93% of adrenocortical adenomas.

5 AIMS OF THE STUDY

- To investigate transcriptional profiles in benign and malignant adrenocortical tumors, through the use of microarray technology.
- To further analyze genetic differences in subtypes of hormone producing adrenocortical tumors and incidentalomas as compared to normal adrenocortical tissue.
- To identify genes that can serve as potential markers for the diagnosis of adrenocortical cancer.

6 MATERIAL AND METHODS

Subjects (I and II)

The studies were performed at the Karolinska University Hospital, in Solna, the Center for Molecular Medicine and the Royal Institute of Technology.

All cases were selected from a cohort of 250 patients who were operated at the Karolinska University Hospital from 1986 to 2004, with the final diagnosis of adrenocortical tumor. All patients were followed for at least 6 months. Median survival in the adenoma group was 96 months and 45 months in the carcinoma group. The median age in the entire group was 63 years (range 29-84 years), in the adenoma group was 59 years (range 29-79 years) and for the cancer group 65 years (range 52-84 years). There were 14 females and 3 males in the adenoma group and 4 females and 4 males in the cancer group. Median tumor size was 4 cm (range 2-6 cm) in the adenoma group, while it was 13 cm (range 9-20 cm) in the cancer group.

The diagnosis of adrenocortical carcinoma was based on histopathological features such as: tumor size, capsular and vascular invasion, mitotic index, necrosis and pleomorphism. All cases were re-evaluated by one of the authors (AH). In cancer, the appearance of metastasis or recurrence helped to establish the diagnosis. Normal adrenal cortex samples were obtained from patients undergoing nephrectomy. Material for the molecular studies was extracted from tissue biopsies.

The samples included were 4 normal adrenocortical samples and 25 sporadic adrenocortical tumors. The adrenocortical tumors were 4 aldosterone producing tumors, 5 cortisol producing tumors, 8 incidentalomas and 8 cancers. A summary of some clinical and sample data is shown in table 1 (II). Five out of eight patients with cancer had metastasis at the time of operation and one got metastasis during the follow up period. Five out of eight patients with cancer received postoperative adjuvant chemotherapy and one of these patients was also treated with radiotherapy for bone metastasis. Only one cancer sample showed a diploid pattern in DNA ploidy analysis while 5 cancer samples were aneuploid. This study was approved by the Ethical Committee at the Karolinska University hospital.

Methods (I and II)

RNA Extraction: A fresh frozen piece of approximately 70 to 100 mg was used for RNA extraction. Representative sections from all specimens were subjected to histopathological evaluation for tumor representativity. All samples were estimated to contain more than 70% tumor cells. Total RNA was extracted using Qiagen mini and midpreps kits for RNA purification. Universal human reference RNA (Stratagene) was used as a common RNA reference for all hybridizations. RNA quantity and quality were assessed by spectrophotometry and Agilent 2100 Bioanalyzer (Agilent Technologies). We used a reference design where each experiment was carried out in duplicates. Tumor samples were labeled with Cy5 and hybridized with Cy3 labeled commercially available Universal Human Reference RNA (UHRR, StratageneTM catalog #740000) on spotted cDNA microarrays containing 30 000 spots.

Microarray production: The microarray consisted of near 30,000 cDNA fragments originating from the sequence verified human clone collection, 97001.V, plates 1-310 (Research Genetics) spotted onto Ultra GAPS slides (Corning). Plasmid preparation was carried out in a 96-plate format from bacterial cell suspensions with Montage 96 Plasmid Prep kit (Millipore) using a Biorobot 8000 (Qiagen). PCR amplifications were performed in 100 µl reaction volumes, and purified using Montage PCR₃₈₄ Filter Plates (Millipore) with a Biorobot 8000 (Qiagen). The purified PCR-products were resolved in 40 µl 30% DMSO and split between a storage plate and a printing plate.

Microarrays were printed with a QArray (Genetix) instrument with 24 SMP2.5 pins (Telechem, Sunnyvale, CA, USA). The 30,000 cDNA fragments, together with 8 copies each of the 23 different Lucidea Universal Scorecard controls (Amersham Biosciences), were spotted in a 25x25 pattern within each block and with a feature center-to-center distance of 170 µm.

Quality of spotted slides was assessed by Syto61 staining (Molecular Probes, Eugene, OR, USA) and random nonamer hybridization. The slides were UV cross-linked at 250 mJ/cm² followed by baking at 75 °C for 2 hours. A complete gene list can be found at www.biotech.kth.se/molbio/microarray.

Target labelling: First strand cDNA target was generated by reverse transcription of 10 µg of total RNA in a 30 µl reaction using 10 µg anchored oligo dT primer (dT20VN, MWG Biotech AG), first-strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂), 0.01 mM DTT, 400 units Superscript II (Invitrogen AB), 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 minutes followed by 2 minutes of incubation on ice for primer annealing before addition of the other reaction components. cDNA synthesis was performed during 2 h at 42 °C. The reaction was terminated by addition of EDTA. The RNA was hydrolyzed in NaOH during a 15 minutes incubation at 70°C followed by neutralization with HCl (final concentrations 20 mM, 150 mM and 150 mM, respectively). The cDNA was purified using MinElute spin columns (Qiagen GmbH) 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-minute incubation at room temperature after which unincorporated ester groups were inactivated through a hydroxylamine treatment (final concentration of 730 mM). Tumour samples were labelled with Cy5 and the reference with Cy3. The labelling reactions were purified using MinElute spin columns. The complete protocol can be downloaded from <http://www.biotech.kth.se/molbio/microarray/index.html>

Quality control of RNA and cDNA: Extraction and labelling quality/quantity were assessed using a nanophotometer and Agilent bioanalyzer 2100.

Hybridizations: The slides were prehybridized in 5X SSC, 0.1% SDS, and 1 % Bovine Serum Albumin (Sigma-Aldrich) for 30 min in 42°C. The hybridisation buffer used for all experiments contained 25% formamide, 5X SSC, 0.1% SDS, 5X denhardtts, 20 µg poly(A) (Sigma-Aldrich), 10 µg COT-1 DNA (Invitrogen), 5 µg tRNA (Sigma-Aldrich). The hybridisations were carried out in hybridisation chambers (Cat # 2551, Corning) for 18 h at 42°C. Post-hybridization washes were carried out according to Corning's recommendations. Hybridizations were performed in duplicates.

Every hybridization is repeated at least twice (replication) in an alternate way (benign-malign tumor) for getting an adequate statistical power. For further details of

aforementioned protocols, please refer to the internet site: <http://www.biotech.kth.se/molbio/microarray/index.html>.

Scanning and Data analysis: The arrays were scanned at 532 and 635 nm with 10- μ m resolution using a G2565BA DNA microarray scanner (Agilent Technologies).

Image analysis was performed in GenePix 5.1 software (Axon Instruments). After removal of bad quality spots (if less than 70% of foreground pixels were below background intensity plus 2 standard deviations in both channels or if signal to noise ratio were below 3 in both channels or if the difference between ratio of medians and regression ratio exceeded 20% in one of the channels) the remaining intensities were print tip LOWESS normalized in the R environment for statistical computing (<http://www.R-project.org>) with the aroma package (<http://www.maths.lth.se/publications>). Levels of expression for every gene are analyzed in separate samples, tumor group (cluster of samples) and in comparison with the common RNA reference. The analysis of these values (expressed as continuous variables) were performed using the Aroma package for normalization and the LIMMA package for statistical testing of significance. B-test for ranking differentially expressed genes was performed on the average of technical replicates on genes represented in all samples using the Limma package (Smyth et al 2004). Clustering was performed with the stats and class packages (<http://www.R-project.org>). Hierarchical clustering of genes for visualisation of expression patterns was performed in MEV (<http://www.tigr.org/>). Functional gene classification was performed according to Gene Ontology (<http://www.geneontology.org/GO.doc.html>). Fisher's exact test was used to evaluate significant overrepresentation of GO terms and chromosomal location.

Quantitative Real Time PCR: In order to confirm the array findings we used a two step TaqMan quantitative real time polymerase chain reaction (qRT-PCR). Tested genes were: ALDH1A1 (Hs 00167445_m1), IGF2 (Hs00171254_m1), USP4 (Hs 00234300_m1) and UFD1L (Hs 00799945_s1).

Synthesis of cDNA from total RNA was done using the High-Capacity cDNA Archive kit^R (Applied Biosystems). We used 0.5-1 μ g of RNA from every sample and added a mix containing: 10x reverse transcription buffer, 25X dNTPs, 10X random primers, nuclease-free water and MultiScribeTM reverse transcriptase (50 U/ μ l) in a final total

volume of 50 μl . The reaction was load into a GeneAmp^R PCR System 9700 thermal cycler (Applied Biosystems). The following conditions were used for the reverse transcription: an initial hold phase of 25°C for 10 mins for incubation, 2 hold phases with 37°C for 60 minutes for reverse transcription, a fourth phase with 37°C for 7 minutes and a final phase with 4°C for 30 seconds. The plate was immediately removed after completing cycling and stored at -15 to 25°C for later use. A 96 well plate was prepared for the TaqMan^R Gene Expression Assays (Applied Biosystems). This is a 5' fluorogenic nuclease assay to quantify the relative abundance of specific transcripts. Every well contained 12.5 μl of TaqMan^R Universal master mix, 1.25 μl of primer plus probmix (Assays on DemandTM 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 done in triplicates and the housekeeping genes in duplicates according to manufacturers recommendations. We always include a non-template control well in every run. Wells were sealed with MicroAmp^R Optical caps. A brief centrifugation was done to spin down the contents and eliminate any air bubbles from the solutions contained in every well of the plate. Plates were loaded into the ABI PRISM 7700 sequence detection system (Applied Biosystems). In order to select a housekeeping gene as reference for the quantification we used the TaqMan^R Human Endogenous Control Plate. With this plate the expression of 11 selected housekeeping genes is evaluated, using a two-step RT-PCR. The plate also features a unique internal positive control (IPC) designed to detect the presence of PCR inhibitors in test samples. We analysed the following genes: internal positive control, 18S rRNA, acidic ribosomal protein, beta-actin, cyclophilin, glyceraldehydes-3-phosphate dehydrogenase, phosphoglycerokinase, b2-microglobulin, beta-glucuronidase, hypoxanthine ribosyl transferase, transcription factor IID, TATA binding protein and the transferring receptor.

7 RESULTS

Pattern of Gene Expression Profiles

A t-test modified with Bayesian statistics was used to rank genes in order of significance of differential expression. A total number of 9670 (I) and 6736 (II) spots/cDNA fragments were present in all samples in at least one technical replicate after quality filtering. In figure 1 the expression profiles of all tumour samples from paper II are visualized in a heatmap dendrogram where the genes have been hierarchically clustered using Euclidean distances and average linkage to get an overview of the general pattern.

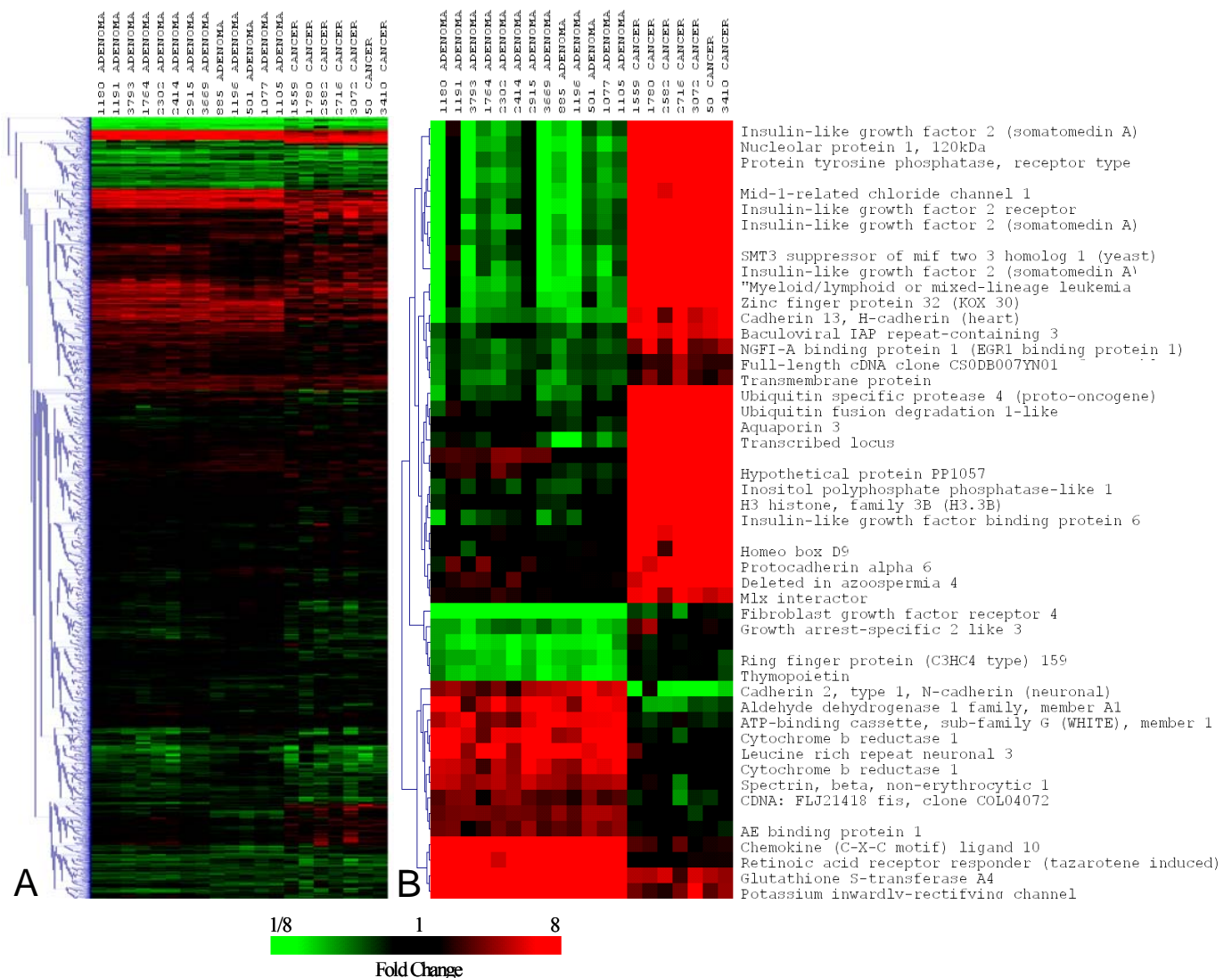


Figure 1a. Heatmap where the entire set of genes have been hierarchically clustered to visualize the general expression patterns. The expression profiles of adenoma samples are homogenous compared to carcinomas, which show greater biological variation based on mRNA levels. **1b.** The 50 most significantly differentially expressed genes according to their B-values.

Pattern Homogeneity: The expression profiles among adenomas are rather homogenous and differ remarkably from the more heterogeneous profiles observed in carcinomas. This heterogeneity was more notorious within different cancer samples, as is illustrated in Figure 1a (I). When clustering samples instead of genes with the same dataset, adenomas and carcinomas were placed in two separate clusters revealing that the malignant phenotype indeed has an expression profile distinguishable from the benign one. Similar result was obtained with three different clustering techniques; hierarchical clustering, k-means clustering and self-organizing maps.

Differential Expression Patterns: Hierarchical clustering, k-means clustering (KMC) and self-organizing maps (SOM) were applied to all gene expression profiles with values in every tissue sample, to reveal that samples could be clustered into two major groups. Figure 2 displays the result of hierarchical clustering using Euclidean distances and average linkage between clusters. Samples clustered into two major groups. One consisted of seven cancer tumours and the other of normal and adenoma samples and, interestingly, also one cancer. This was cancer sample number 2931 (table 1 paper II). Patient 2931 is still alive 67 months after surgery. Histopathological re-assessment showed that the diagnosis of cancer is uncertain. As our aim was to identify the most important genes in the different subtypes, we decided to omit this case in the subsequent comparative analysis. Similar results were obtained with Pearson correlation as distance metric in the hierarchical clustering and using two other unsupervised methods; KMC and SOM (II).

Interestingly, this case (2931) also showed gene expression levels similar to the “normal or benign” pattern in the quantitative RT-PCR for IGF2 and ALDH1A1, shown in figure 7 (II).

A t-test moderated with an empirical Bayesian statistic was applied to test for differential expression. Six comparisons were performed, each of the four tumour subgroups against all the other samples, one comparison between adrenal Cushing’s syndrome and aldosterone producing tumors and one between cancers and adenomas. The cancer sample with uncertain diagnosis was omitted from the analysis in order to eliminate any possible confusing variable or misclassification bias.

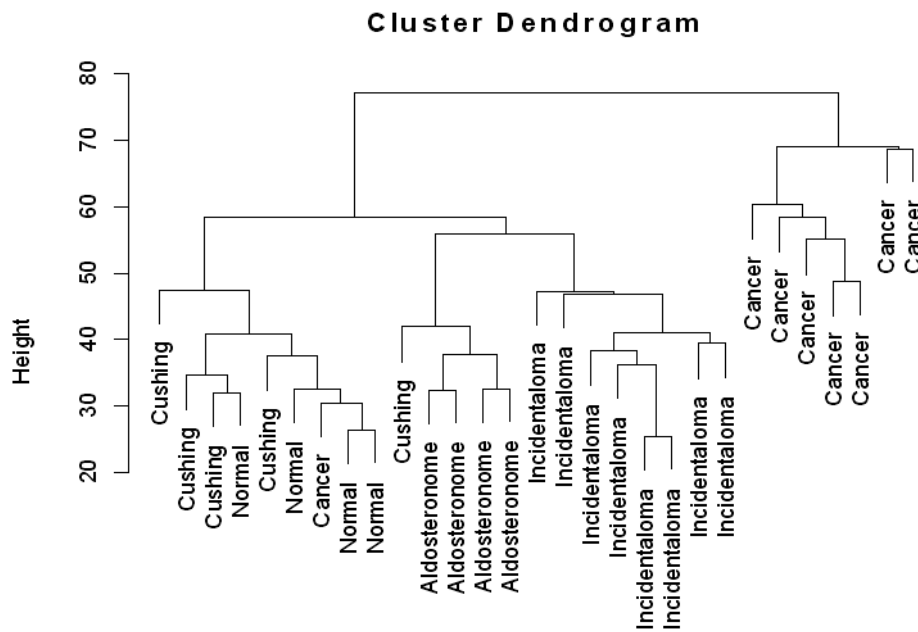


Figure 2. Hierarchical cluster of all tumors based on the gene expression profiles of the 6736 features present in all tumors

Four hundred eight genes were differentially expressed comparing cancer to all other samples using the criteria for statistical significance described in material and methods (II). Features included in the test were required to be present in all cancers and in at least eleven out of the 20 other samples because of the variation among cancer samples and also to increase the chance of finding potential biomarkers for cancer. The 40 most significantly differentially expressed transcripts are displayed in figure 3 (II). Comparing each functional subtype of adenomas to all other samples based on the 6736 genes with values in all samples, the number of statistically significant features was 182 for aldosterone producing tumors, 68 for incidentalomas and none for cortisol producing tumors. However there was some individual gene expression levels differentially expressed between cortisol and aldosterone producing tumors (II).

Functional Classification of the Genes: This was explored through the use of Gene Ontology annotation for the most differentially expressed genes in each clinical group (I). The majority of up regulated genes in carcinomas appeared to have a predominant nuclear (GO 5634) location ($p < 0.004$) while the down regulated genes had their highest representation in the cytoplasm (GO 5737) and plasma membrane (GO 5886) location

($p < 0.05$). Other genes with altered transcript levels in cancer were predominantly related to specific cellular functions such as mitosis, cell adhesion and extracellular component (II).

Quantitative Real Time PCR: The less variable housekeeping gene across all samples in the TaqMan^R Human Endogenous Control Plate, was 18S ribosomal RNA. Therefore we selected this gene as a reference for all consecutive gene quantifications. Quantitative RT-PCR for the tested genes ALDH1A1, IGF2, USP4 and UFD1L showed a good correlation with the values obtained by the microarray technique (II).

Specific Genes: We found at least two specific genes which could be useful markers for adrenocortical cancer. IGF2 which was upregulated and ALDH1A1 which was down regulated in all carcinomas, except for the uncertain cancer case 2931 (II). The expression levels of these genes were confirmed by quantitative RT-PCR technique figure 7 (II). Other genes that could be potentially useful as markers for adrenocortical cancer are listed in table 2 (I), and in figures 3a, 4a and 4b (II).

8 DISCUSSION

It is a major problem to distinguish adrenocortical adenomas from carcinomas. Previous studies have demonstrated that adrenocortical carcinomas display specific and more numerous chromosomal and/or genetic alterations than adenomas, which could suggest a characteristic malignant genotype. In the present study we showed that with cDNA array technology different subtypes of functional adenomas and carcinomas can be distinguished based on their specific molecular signature.

Expression profiles were shown to be clearly different between adenomas and carcinomas, with a homogeneous pattern among the adenomas and a heterogeneous pattern in the carcinomas. Functional classification of the up regulated genes in carcinomas showed predominantly a nuclear location whereas the down regulated genes displayed cytoplasmic and plasma membrane location ($p < 0.05$). Regarding chromosomal localization (I) of the most significantly up regulated genes in carcinomas, chromosomes 12 and 5 had a major number of involved loci, while chromosomes 2 and 1 had the major number of involved loci regarding the down regulated genes. These findings are consistent with earlier CGH studies.

Upregulated Genes in Cancer/Adenoma

The two most significantly up regulated genes (I) were the ubiquitin related genes: ubiquitin specific protease 4 (USP4) and ubiquitin fusion degradation 1-like (UFD1L). Both genes were up regulated at least 40 times in carcinomas as compared to adenomas. USP4 is a proto-oncogene related to a group of deubiquitinating enzymes located mainly in the cell nucleus. The over expression of the USP4 gene is associated with malignant transformation of small cell adenocarcinoma cells in vitro and in vivo. The second ubiquitin related gene is the UFD1L. The product of this gene is involved in the degradation of ubiquitinated proteins and it is probably involved in chromatin remodeling (Amati et al 2003). However the precise biochemical role of UFD1L in human cells remains to be further elucidated.

An important group of over expressed genes in our study (I and II) includes members of the insulin-like growth factor system (IGF2, IGF2R, IGFBP3 and IGFBP6). These genes are up regulated in carcinomas as compared to adenomas. One of these, the insulin-like growth factor 2 (IGF2) is the most consistently up regulated gene in

adrenocortical carcinomas (Giordano et al 2003, de Fraipont et al 2005). The IGF2 has an important role as an autocrine regulator of cell proliferation and apoptosis (Coulter et al 2005). The over expression of IGF2 has also been associated with the progression of several other cancer types such as gastric cancer (Pavelic et al 2003), breast cancer (Lee et al 2003), hepatocellular (Li et al 2004, Tsujiuchi et al 2004) and colorectal cancer. Moreover, human antibodies directed to IGF2 protein have been shown to inhibit prostate tumor growth in vivo. High plasma levels of IGF2 and low plasma levels of the insulin-like growth factor binding protein 3 (IGFBP3) are associated with a high risk of endometrial cancer (Oh et al 2004). The insulin-like growth factor 2 receptor (IGF2R) is considered a tumor suppressor gene. This gene encodes for a non-mitogenic receptor, which targets the IGF2 to the lysosomes for degradation and thereby it controls the IGF2 proliferative activity. The up regulation of the IGF2R gene in the carcinomas is somewhat contradictory to its role as a tumor suppressor gene. However, there are studies showing aberrant transcript levels associated with different IGF2R mutations related to the development of hepatocellular and gastric carcinoma as well as adrenocortical tumors. The insulin-like growth factor binding protein 6 (IGFBP6) is also over expressed in all the carcinomas, a finding consistent with observations in testicular and papillary thyroid carcinoma. The insulin-like growth factor binding protein 3 (IGFBP3) showed a lower level of up regulation than other IGF related genes among the carcinomas (Renahan et al 2004). Its product functions as a major carrier protein for circulating IGF2 and thereby blocking its activity. Negative correlation has been reported between cancer risk and IGFBP3, although these data have been challenged by other groups. IGFBP3 over expression may be induced by the COX-2 inhibitor Celecoxib, leading to apoptosis in vitro (Levitt et al 2004, Deal et al 2001). An additional instrument could be bioradionuclide imaging with the product of these genes, in order to localize the tumor. In this case, a radioactively labeled antibody towards the IGF2 receptor would be one possible choice, which also could reveal the localization of small metastasis not visible in ordinary radiological scans. For this purpose it would be interesting to assess the gene expression profiling on the metastasis of adrenocortical cancers.

Our results are similar to previous studies mainly in the categorization of adrenocortical carcinomas with the gene marker IGF2 (de Fraipont et al 2005, Giordano et al 2003). In one study (Giordano et al 2003) the expression level of IGF2 allowed to distinguish 90% of adrenocortical cancers while in other study (de Fraipont et al 2005) they could

discern at least 75%. Additionally they found a downregulated gene in cancer, HSD3B1. This gene was downregulated in 81% of the cancer cases while it was upregulated in 93% of the adenomas. The authors were also able to determine a set of genes useful for discriminating recurring from nonrecurring carcinomas. However their study did not contain any aldosterone producing tumors or normal cortical tissue.

We specifically focused on the differential expression of membrane receptors since our main interest was addressed in genes which products eventually could serve as drug targets or biomarkers for bioimaging. The main needs of diagnostic markers are firstly to detect the tumor and secondly to distinguish between cancer and adenoma. Four growth factor receptors were up regulated in cancer, IGF2R, FGFR1, FGFR4 and MET. Two of these, IGF2R and FGFR4, could be used for classification of carcinomas based on gene expression profile without false positives, or worse, false negatives. MET misclassifies cancer 3072 and FGFR1 cancer 2716. For identification aldosteronomes VEGFB is the best candidate. There was no receptor suitable for classification of incidentalomas or cortisol producing tumors.

Downregulated Genes in Cancer/Adenoma

Down regulated genes in carcinomas (I) as compared to adenomas were among others, the chemokine (C-X-C motif) ligand 10 (CXCL10), which is involved mainly in cell communication and morphogenesis. CXCL10 encodes an angiogenesis and inflammatory modulating cytokine. In contrast CXCL10 has also been found over expressed in colorectal cancer.

Other down-regulated genes such as the retinoic acid receptor responder (tazarotene induced) 2 (RARRES2), the aldehyde dehydrogenase 1 family, member A1 (ALDH1A1), the Cytochrome b reductase 1 (CYBRD1) and the Glutathione S-transferase A4 (GSTA4) are mainly involved in cell metabolism. Cadherin 2, type 1, N-cadherin (neuronal) (CDH2) encodes a cell adhesion molecule. This gene has been reported epigenetically silenced in pancreatic cancer (Hagihara et al 2004), thereby decreasing its expression. This could potentially be the underlying mechanism for the down regulation observed in our tumor samples. Other down regulated genes in cancer as compared to benign tumors are listed in table 2 (I).

Functional Tumors

Regarding functional tumors, we expected differences in gene expression patterns related to steroid hormone synthesis. The key genes were unfortunately not included in the array, but other interesting genes involved in intermediate metabolism were; the oxysterol binding protein (OSBP) was up regulated in aldosterone producing tumors exclusively. This gene is involved in sterol transport from lysosomes to the nucleus downregulating the expression of other genes such as LDL-receptor, HMG-CoA reductase and HMG synthetase, involved in lipid synthesis. Another interesting gene called mevalonate kinase (MVK) was also also exclusively upregulated in aldosterone producing tumors as compared to cortisol producing tumors. This gene is directly related to sterol (cholesterol) synthesis. On the other hand a similar gene related to lipid metabolism called fatty acid desaturase 2 (FADS2), was overexpressed in cortisol producing tumors. These are three examples how some genes related to cholesterol biosynthesis could be useful to distinguish these two entities, however further investigation to clear their role in the pathophysiology of these diseases is necessary.

Other interesting genes found (II) were VEGFB, homeobox D8, APC DD1 and adenosine deaminase (ADA). They were overexpressed in aldosterone producing tumors and downregulated in cortisol producing tumors. These genes could be potentially useful to distinguish these two entities. There were genes overexpressed in cortisol producing tumors and downregulated in aldosterone producing tumors. Examples of these were the Inositol 1,3,4-triphosphate 5/6 kinase (ITPK1), occludin, the insulin growth factor binding protein 3 (IGFBP3), ubiquitin thiolesterase 1 (UCHL1), tumour necrosis factor receptor superfamily member 25 (TNFR SF25), and the V-abl Abelson murine leukemia viral oncogene homolog 1 (ABL1).

In our study we found four hundred eight genes differentially expressed comparing cancer to all other samples (I and II). Additionally, comparing each functional subtype of adenomas to all other samples, the number of significant genes was 182 for aldosterone producing tumors, 68 for incidentalomas and none for cortisol producing tumors. However there were some individual gene expression levels that could differentiate between cortisol and aldosterone producing tumors.

Through the use of cDNA arrays different subtypes of functional adenomas and carcinomas may be distinguished based on their specific molecular signature. The

biological and etiological importance of the up and down regulated genes in the corresponding groups or subtypes of adrenocortical tumors has to be further analyzed. Identification of these genes may enhance our understanding of the molecular biology of adrenocortical tumor development and its functional phenotype, and may create possibilities for new diagnostic and prognostic tools.

9 CONCLUSIONS

- Through the use of cDNA arrays the transcriptional profiles may clearly distinguish adrenocortical adenomas from carcinomas based on their specific molecular signature.
- Several genes were identified which were differentially expressed in benign adrenocortical tumor subtypes and incidentalomas as compared to normal adrenocortical tissue.
- Many genes were identified that can be potentially useful for the diagnosis of adrenocortical cancer.

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