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How the new tools to analyze the human genome are opening new perspectives: The use of gene expression in investigations of the adrenal cortex

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Abstract

With the promise of state-of-the-art molecular technologies and the tools provided by the human genome project, a number of investigators are trying to identify molecular targets of adrenocortical tumorigenesis. One path in this endeavor was the identification by positional cloning of genes that are mutated in rare adrenocortical tumors. The subject of this article is an update of the results of experiments in the second path that was followed by us and others: that of using genome-wide expression analysis of adrenocortical cells in normal and various disease states. Transcriptomic analysis is a rapidly evolving technology; this article summarizes some data on the adrenal cortex and points out how these new technologies can be used in the identification of important genes and molecular pathways in both normal and diseased adrenal cortex.

Keywords

Adrenal cortex; Microarrays; Serial analysis of gene expression (SAGE); Oligonucleotides; Complementary DNA (cDNA); Messenger RNA (mRNA); Gene expression; Adrenal hyperplasia

1. Introduction

Clonal composition analyses and comparative genomic hybridization (CGH) experiments have suggested that adrenocortical tumorigenesis is a multistep process that follows a sequence of genetic alterations leading to malignancy [1-4]. However, the rarity of adrenal cancer compared to the relative frequency of benign lesions suggests that either the accumulation of mutations most often does not lead to cancer, or that there are additional pathways to adrenal carcinogenesis. Just like CGH in the past allowed an instant and clear comparison of the DNA from tumor and normal tissues, the examination of the adrenal transcriptome allows for the detection of expressed genes in normal and abnormal cells and provides an even higher resolution image of the differences between physiology and pathology. Expression analysis does not replace DNA genomic approaches; it supplements other tools developed by the human genome project and should be used in conjunction with such techniques as CGH and traditional cytogenetics. For example, expression arrays, due to their higher resolution, can often lead to the identification of DNA alterations that would otherwise have been missed by molecular cytogenetics. At the end, an analysis of the transcriptome can only be meaningful when it is compared to genomic and proteomic studies. In this chapter, we summarize the latest findings

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in adrenocortical whole-genome expression analysis; the relatively few studies are quite similar in their results and provide a solid basis for the next step, that of the proteomic analyses.

2. Methods: approaches in the study of adrenal gene expression

Currently, transcriptional profiling of any tissue is performed applying various types of microarrays and the alternate technology of generating libraries of expressed sequence tags (ESTs). An advanced expansion of EST libraries, especially in terms of high-throughput and transcript quantitation, is serial analysis of gene expression (SAGE). SAGE is based on generating, cloning, and sequencing concatenated short sequence tags, each representing a single transcript derived from mRNA from target tissue [5]. Analysis of the transcriptional data gained by the above methods is most commonly performed using clustering algorithms that group genes and samples on the basis of expression profiles, and statistical methods scoring the genes on the basis of their relevance to the clinical manifestations.

The method of choice into consideration for global expression profiling depends on several factors including technical, labor, price, time and effort involved, and, most importantly, the type of information that is sought. When comparing microarrays to EST libraries, an appreciable advantage of the later is its inherent ability to identify transcripts without prior knowledge of the genes' coding sequences, hence its important potential for cloning and sequencing novel transcripts and genes. On the other hand, recent technical advances in the development of expression arrays, their abundance and commercial availability, and the relative speed with which analysis can be done, are all factors that make arrays more useful in routine applications. In addition, array content can now be readily customized to cover from gene clusters and pathways of interest to the entire genome: some studies examine series of tissue specific transcripts and/or genes known to be involved in particular pathology; others directly use arrays covering the whole genome.

Another factor that needs to be considered prior to embarking on any high-throughput approach is whether individual or pooled samples will be investigated. Series of pooled samples reduce the price, the time spent and the number of the experiments down to the most affordable. Investigating individual samples, however, is important for identifying unique expression ratios in a given type of tissue or cell.

Two EST libraries and five different microarray studies on human adrenals have been published so far. In the first library, normal, nondiseased human adrenals are profiled [6]. For this study, RNA from two adult individuals was used. Totally 20,626 ESTs were assembled into 9175 gene clusters (3979, 3074, and 4116 clusters in hypothalamus, pituitary, and adrenal glands, respectively). Of these clusters, 30.3% corresponded to known genes, 44.8% to dbESTs, and 24.3% did not correspond to known coding sequence. One of the main outcomes of this study was cloning and sequencing of 200 previously unknown transcripts, 97 of which were adrenal-specific.

The second adrenal global profiling study compared two SAGE libraries – one from a normal adrenal gland and another from a form of bilateral adrenocortical hyperplasia–primary pigmented nodular adrenocortical disease (PPNAD), both from adolescent females [7]. Fourteen thousand eight hundred and forty-six and 16,698 unique mRNAs from the normal adrenal tissue and PPNAD, respectively, were catalogued and their expression quantified. Compared to the previous study, this one found only 842 (~ 6% of the total) sequences that did not match any known expressed sequences and thus corresponded to novel genes and transcripts. Interestingly, however, most unknown transcripts were also among the most abundant or most adrenal-specific transcripts, indicating –like the previous study – that there are quite a number of adrenal-specific genes with unknown function and with limited

expression elsewhere. Our laboratory is now attempting to clone the most significant adrenal-specific sequences from this library.

The first of the microarray studies that was published addressed primarily normal adrenal tissue and compared fetal and adult adrenal expression [8]. Total RNA from 18 fetal (between 15 and 20 weeks of gestation) and 12 adult adrenal glands were studied in this analysis. The RNA samples were pooled and hybridized to five independent microarrays containing between 7075 and 9182 cDNA elements.

Our laboratory used the same approach to study another form of adrenal hyperplasia-corticotropin (ACTH)-independent, bilateral adrenocortical hyperplasia or massive macronodular adrenocortical disease (MMAD); these data were recently published by Bourdeau et al. [9]. This analysis was performed on eight tissues (three of them from patients with food-dependent Cushing syndrome) and compared them with the expression profile of pooled adrenal gland RNA that was obtained from 62 healthy individuals and is commercially available. The arrays utilized for this study contained approximately 10,000 human PCR-amplified cDNA clones and were made at the National Cancer Institute (Bethesda, MD, USA) core facility. As a criterion of differential expression, a cut off of two along with alteration in at least 75% of the patients was applied. According to these stringent criteria, 82 and 31 genes were found to be consistently up- and downregulated, respectively.

Two other microarray studies compared adrenocortical adenomas and carcinomas [10,11]. The first among them generated transcriptional profiles of 11 carcinomas, four adenomas, three normal adrenal cortices, and one macronodular hyperplasia using Affymetrix HG U95Av2 oligonucleotide arrays that represented 10,500 unique genes. The second investigated a series of 230 candidate adrenal-specific genes, an “adrenochip”, that included 187 cancer-related genes (including genes encoding cell cycle control proteins, growth factors, growth factor receptors, transcription factors, cell adhesion molecules, and proteins involved in cell invasion, angiogenesis, and chemoresistance that were previously shown to be expressed in the adrenal and were suspected to do so), 34 adrenal cortex-specific genes (including genes encoding hormone receptors, components of the cAMP signaling pathway, steroidogenic enzymes, and components of the IGF2 system), and nine control genes, most of them classic house-keeping sequences. This gene set was examined in a series of 57 well-characterized human sporadic adrenocortical tumors (33 adenomas and 24 carcinomas). Quite amazingly and despite their different design these two studies revealed highly concordant results.

Finally, a recent study attempted to catalogue the genes expressed in aldosterone-producing adenomas and, more specifically, to determine the degree of alteration of G-protein coupled receptors expression, when compared to the normal adrenal cortex [12]. For this analysis RNA from an adult adrenal and a pool of three RNA samples from aldosterone producing adenomas were hybridized to an Affymetrix human HG-U133+2 oligonucleotide microarray set containing 54,675 probe sets representing approximately 40,500 independent human genes.

A requirement of all high-throughput approaches is confirmation of findings (expression level of a given gene/sequence) by other methods. A select group of genes are tested usually: these genes are picked from the series of sequences that were analyzed either because they were found to have significant changes or due to their particular interest with regards to their expression in adrenocortical tissue or their previously identified relationship to pathology or developmental stage. The confirmation process attempts to support the findings on three different levels:

- reliability of the high-throughput experiment – for this purpose the same samples examined by the EST libraries or microarrays are used;

- trustfulness of the observations in general – to achieve that, larger number of samples are examined, assessment of which by high-throughput approaches is often unaffordable (price-or labor-wise);
- verification of the expression changes at the protein level.

All the above-described studies applied at least one, and occasionally two or even three conventional methods to confirm the high-throughput findings. Most utilized the recently evolved quantitative real time reverse transcription PCR (Q-RT-PCR), although a classic Northern blot could also be used. The latter, unfortunately, requires several fold higher amounts of mRNA, which is often hard for adrenocortical studies. For verification at the protein level immunohistochemistry (IHC) and Western blot are the two most commonly chosen techniques. IHC is not quantitative but has the advantage of allowing for the observation of the exact localization of a signal within a cell (cytoplasmic versus nuclear) and the tissue (identifying histologically the tissue that is stained). Modern Western blot methods require smaller amount of protein lysate than older techniques, and have the advantage of offering high resolution quantitation of expression without the use of radioactivity. Application of these confirmation approaches in adrenal high-throughput expression studies has shown a high degree of reliability for both microarrays and EST libraries. Recent reviews have pointed out the significance and reproducibility of these data [13,14].

3. Notes

3.1. Genes expressed in normal adrenals

The global expression profile of normal human adrenals reflects this steroidogenic tissue's functional characteristics [6,7]. Genes involved in fundamental biological processes, including protein synthesis and processing, energy metabolism, and cell structure, are prevalent. And accordingly, the most adrenal-specific gene cluster included in all studies genes involved in steroidogenesis such as steroidogenic acute regulator (*STAR*), 11-beta-hydroxylase (*CYP11B1*), 17-alpha-monooxygenase (hydroxylase) (*CYP17A1*), 21-monooxygenase (hydroxylase) (*CYP21A2*), 3 beta-and steroid delta-isomerase 2 (*HSD3B2*) and others enzymes. Developmental genes with known function in the adrenal cortex were also among the most adrenal-specific expressed transcripts, including the nephroblastoma overexpressed gene (*NOV*), chromogranin B (*CHGB*), and delta-like 1 homolog (*DLK1*)).

The overall analysis of the existing studies revealed *STAR* as the most highly expressed, tissue-specific gene for normal human adrenal cortex. It is noteworthy that a number of adrenal ESTs represented rare transcripts of otherwise ubiquitously expressed genes. These included β 2-microglobulin (*B2M*), plasminogen (*PLG*), myosin light polypeptide 6 (*MYL6*), and many more. For these genes, more than one transcript has been identified, and the adrenal preferential expression of this transcript did not correspond to the most common and ubiquitously expressed sequence. This finding is suggestive of an extensive presence of alternatively spliced transcripts of known genes in adrenal cells [15].

In our SAGE library, a high proportion of the identified adrenal-specific ESTs did not correspond to known genes and thus represent potentially novel genes or alternative transcripts.

To assess the degree of differentiation of the adrenocortical cells, criteria for tissue specificity previously employed for SAGE libraries by Velculescu et al. [16], were applied. In brief, the proportion of the transcripts present at 10 and more copies/cell and expressed at very low levels or absent in all other tissues, was assessed. This percentage was 1.71% of the transcripts in the normal adrenal tissue library, ranking adrenal expression among the highest in specification for normal human tissue-specific gene expression; (in the prostate, for example, it stands at 0.05%) and suggesting a high level of differentiation for adrenocortical cells.

The age-related differentiation and functional development of the adrenals have been studied by comparing the gene expression profiles of human fetal adrenal and adult adrenal gland. Sixty-nine transcripts were found to have a greater than 2.5-fold difference in expression between fetal and adult adrenals [8]. Interestingly, the vast majority of these transcripts have not been studied before with regard to adrenal function. The largest differences were observed for transcripts that encode insulin-like growth factor 2 (*IGF2*) (25-fold higher in fetal adrenal) and 3beta-hydroxysteroid dehydrogenase (*HSD3B*) (24-fold higher in adult). The enhanced fetal expression of *IGF2* transcripts (second only to the liver) is well documented [17]. This finding probably reflects the ability of the adult adrenal to secrete aldosterone as well as large quantities of both cortisol and dehydroepiandrosterone (DHEA) sulfate, while the mid-gestational fetal adrenal secretes substantial amounts of DHEA sulfate but minimal amounts of cortisol or aldosterone. It is noteworthy that the vast majority of the differentially expressed transcripts have not been studied before with regard to adrenal function.

A group of *IGF2*-related transcripts that were expressed higher in the fetal adrenal were those related to growth and development. The fact that adrenal *IGF2* expression as well as the expression of other growth-related transcripts drops dramatically after birth has led to the hypothesis that *IGF2* could play an important role in the unique functions and/or hyperplasia of the fetal adrenal gland [18]. The size of the fetal adrenal (similar to the kidney at 18 weeks) has caused investigators to search for altered growth factor expression that might regulate its hyperplasia. In addition, growth factors have been studied as potential paracrine regulators of steroidogenic capacity of the fetal adrenal. In vitro studies have shown that *IGF2*, acting through the *IGF1* receptor, increases expression of the enzymes in steroid hormone biosynthesis [19].

Another group of transcripts that were found highly expressed in the fetal adrenal were those involved in de novo cholesterol biosynthesis. Human fetal adrenal has been found to possess the greatest ability to produce cholesterol of all fetal tissues [20]. In addition, elevation in the expression of low density lipoprotein (LDL) receptor was observed. A number of studies support the idea that the fetal adrenal rapidly metabolizes any available circulating LDL to provide cholesterol for steroidogenesis [21,22]. Transcripts related to cellular immunity and signal transduction were preferentially expressed in the adult adrenal. In addition, this study outlined two potentially important for the adrenal function genes with differential expression between fetal and adult adrenal:

- *NGFIB* – an orphan nuclear receptor that appears to be an important regulator of transcription of certain of the steroid-metabolizing enzymes [23];
- *KIAA0018* – one of the most highly expressed transcripts in the fetal adrenal gland. GenBank searches indicated that the only protein with structural similarity to KIAA0018 is the plant enzyme, DIMINUTO/DWARF1, which is involved in plant steroid biosynthesis and critical for plant reproduction [24].

The role of these and others highly expressed in the normal adrenals transcripts identified by using microarrays and other high-throughput techniques warrant further study.

3.2. Genes expressed in adrenal hyperplasia

The two forms of ACTH-independent cortisol-producing bilateral adrenal hyperplasia that have been comprehensively profiled so far are PPNAD and MMAD. PPNAD is characterized by small to normal-sized adrenal glands containing multiple small cortical pigmented nodules [25,26]. PPNAD may occur in an isolated form or associated with a multiple neoplasia syndrome, the complex of spotty skin pigmentation, myxomas, and endocrine overactivity, or Carney complex (CNC), in which Cushing's syndrome is the most common endocrine manifestation [27]. PPNAD, isolated or associated with CNC, is caused mostly by inactivating

mutations of the *PRKARIA* gene, which codes for regulatory subunit type I of protein kinase A (PKA), the main mediator of cAMP signaling in mammals [28-30]. The PPNAD SAGE library was generated from PPNAD from a patient carrying a germline inactivating mutation of the *PRKARIA* gene and compared with that of control healthy adrenal gland from an age- and gender-matched individual.

MMAD is a rare condition in which cortisol secretion may be mediated by non-ACTH circulating hormones such as gastric inhibiting polypeptide (GIP) (leading to food-dependent Cushing's syndrome), vasopressin, catecholamines, luteinizing hormone, serotonin, angiotensin-II or leptin. MMAD may develop during the first year of life with McCune–Albright syndrome, whereas the majority present as the isolated type in the fifth decade of life [31,32]. Although the most frequent clinical presentation of MMAD is CS, cases with excess gluco-mineralocorticoid, cortisol-estrogen, and -androgen secretion have also been reported [33-35]. Usually, computed tomography reveals clear enlargement of both adrenal glands. Histological examination is characterized by nonpigmented nodules composed of two types of cells, those with a clear cytoplasm (lipid-rich) that form cordon nest-like structures, and others with a compact cytoplasm (lipid-poor) that form small nest or island-like structures [36,37].

Analysis of the global expression profiles in these two adrenal hyperplastic tissues showed significant differences, but also, some important similarities [7,9]. An interesting finding was the involvement of the Wnt signaling pathway in both types of hyperplasia. The expression levels of several members of the pathway were found significantly elevated; these included catenin (cadherin-associated protein)-like 1 (*CTNNAL1*), disheveled, dsh homolog 2 (drosophila; *DVL2*), casein kinase 1 (*CSNK1E*), axin 1 (*AXIN1*), catenin- β 1 (*CTNNB1*), WNT1-inducible signaling pathway protein 2 (*WISP2*), and glycogen synthase kinase- β 3 (*GSK3B*).

Wnt signaling transduction pathway regulates many important cellular and developmental processes, including proliferation, cell-to-cell adhesion, cell fate decisions and differentiation. The involvement of this pathway in adrenocortical tumorigenic processes was recently supported by finding somatic activating mutations in beta-catenin (*CTNNB1*) in as high as approximately 30% in both adrenal adenomas and carcinomas [38]; all these mutations were observed in tumors with abnormal β -catenin accumulation.

Another finding in common for the two hyperplastic tissues was the increased degree of expression of PKA-related genes, specifically *PRKAR2B*, which is known to be involved in adrenocortical tumors [39,40]. This observation was consistent with upregulation of the PKA signaling pathway in human tissues bearing *PRKARIA*-inactivating mutations, as well as in *Prkar1a*-deficient mice [41-43].

Finally, another similarity between the two hyperplastic conditions was the increased presence of transcripts involved in cell cycle, adhesion, growth- and proliferation-regulating signal transduction pathways, suggesting an elevated potential for growth and proliferation for these lesions.

An important group of genes that were found inversely regulated in PPNAD and MMAD were those involved in steroidogenesis, and particularly, in cortisol production. In PPNAD, as expected from a high cortisol producing tissue, *CYP11B1*, *CYP17A1*, *CYP21A2*, and *HSD3B2*, were overexpressed. In contrast, the MMAD expression levels of *CYP11B1*, *CYP17A1* and *CYP21A2* were found downregulated, a fact that was confirmed by immunohistochemistry studies [44] and was proposed to reflect the relatively low efficiency with which MMAD cells produce cortisol compared to the volume of the tissue and the degree of their hypertrophy in EM studies [25].

3.3. Genes expressed in adrenal adenomas and carcinomas

Studies on individual genes and pathways that may be differentially expressed in adrenal tumors have repeatedly found several genes/gene clusters activated or repressed. One of the most commonly present alterations in the adrenal carcinomas was *IGF2* overexpression. It has been identified in as many as 85% of the carcinomas in examination on selected markers for adrenal malignancy [45,46]. In a search for related and other genes involved in adrenal carcinogenesis, two different microarray studies examining a total of 75 adrenocortical tumors have been published. The first among them generated transcriptional profiles of 11 carcinomas, four adenomas, three normal adrenal cortices, and one macronodular hyperplasia using Affymetrix HG U95Av2 oligonucleotide arrays representing 10,500 unique genes [10]. The other one covered a series of 230 adrenal-specific candidate genes (“adrenochip”) that included 187 cancer-related genes (including genes encoding cell cycle control proteins, growth factors, growth factor receptors, transcription factors, cell adhesion molecules, and proteins involved in cell invasion, angiogenesis, and chemoresistance), 34 adrenal cortex-specific genes (including genes encoding hormone receptors, components of the cAMP signaling pathway, steroidogenic enzymes, and components of the IGF2 system), and nine control genes [11]. Using this gene set in a series of 57 well-characterized human sporadic adrenocortical tumors (33 adenomas and 24 carcinomas) not only confirmed the *IGF2* increased transcription levels in carcinomas but also identified the cosegregation of additional overexpressed and related genes, forming the so-called *IGF2* cluster. Interestingly, overexpression of this cluster, combined with underexpression of a second cluster (steroidogenesis cluster) appeared to be extremely powerful discriminator between adrenal adenomas and carcinomas.

The *IGF2* cluster contained eight genes that encode growth factors (*IGF2* and transforming growth factor beta (*TGFB2*)), growth factor receptors (fibroblast growth factor receptor type 1 (*FGFR1*), *FGFR4*, macrophage stimulating 1 receptor (*MSTIR*), and *TGFBRI*), as well as *KCNQ1OT1* (also known as *LIT1*, that is long QT internal transcript1) and glyceraldehyde-3-phosphate dehydrogenase (*GAPD*), a presupposed housekeeping gene. It is noteworthy that fibroblast growth factor receptors *FGFR1* and *FGFR4* are the two of the family of four most strongly expressed in the adrenal cortex tyrosine kinase FGF receptors [47,48]. They bind fibroblast growth factors FGF-1 and FGF-2, which are known as the most powerful mitogens for adult steroidogenic adrenocortical cells [49] as well as for the human adrenocortical tumor cell line NCI-H295R [50]. They are also known to stimulate the proliferation of endothelial and mesenchymal cells. Thus, the overexpression of the above two *FGFRs* in adrenocortical cancers is likely to reflect their participation in the cellular increased proliferation and vascularization of this tissue during tumorigenesis.

The second cluster (steroidogenesis cluster) contained 14 genes, six of them encoding proteins directly involved in the steroid biosynthetic pathway: *STAR*, *CYP11A*, *HSD3B1*, *CYP11B1*, *CYP21A2*, and *CYP17*. It also contained protein phosphatase 1A (*PPM1A*), *S100B* (S100 calcium-binding protein, β -chain), glypican 3 (*GPC3*), cAMP response element modulator (*CREM*), retinoblastoma 1 (*RBI*), and *TGFBRI3*. It is noteworthy that the correlation between the level of expression of these different enzymes in each individual tumor and their steroid secretion profile was not trivial. Why the steroidogenesis cluster is underexpressed in adrenal malignant tumors remains to be clarified, but one hypothesis is that it reflects the overall decreased level of tissue-specific functional differentiation that is commonly associated with advanced stages of carcinogenesis.

This study also identified 14 genes, whose expression levels were able to strongly predict the probability of recurrence for the adrenal carcinomas. Indeed, the group of recurring carcinomas could be separated from the group of nonrecurrent tumors on the basis of overexpression of six genes, including *ISGF3G*, *IL2RG*, *GZMA*, *PTPN2*, *ITGB2* and *ATF1* and underexpression of eight genes, including *GAPD*, *ACTG1*, *TLN1*, *PRKCSH*, *VIL2*, *ECE1*, *CDKN2A* and *FOS*.

However, this latter group of carcinomas was not large enough to provide significant data; additional validation in a larger sample set and by other techniques is required.

The second microarray study on adrenal adenomas and carcinomas utilized the Affymetrix HG U95Av2 chip with 10,500 unique human genes. One of the most essential finding was again the elevated expression of *IGF2*: significantly increased expression of *IGF2* was observed in 10 of 11 (90.9%) adrenocortical carcinomas (compared to the mean of the normal cortex/adrenal adenomas cohort) for all three probe sets representing *IGF2* gene in the U95A expression chip. Only one carcinoma did not show increased *IGF2* expression and this was one of the two carcinomas identified by all the statistical means as outliers in that study.

A second important finding in agreement with the previous studies was the significant elevation of *FGFR1* in adrenocortical carcinomas as opposed to adenomas. *FGFR1*, which was represented four times on the array, showed 5.6-, 2.1-, and 2.8-fold increased expression in three of the probe sets (with statistical significance $P = 0.00009$; $P = 0.0003$; and $P = 0.003$, respectively). In contrast, no other receptor tyrosine kinases were found to be differentially expressed. These included *EGFR*, *ERB-B2*, *HER3*, *PDGFRA*, *PDGFRB*, *KIT*, *FGFR4*, *IGF1R*, *IGFR2*, *INSR*, *ESR1*, *ESR2*, *PGR*, and others.

Applying hierarchical clustering and principal component analysis on the three diagnostic groups (normal cortex, adrenocortical adenomas plus micronodular hyperplasia, and adrenocortical carcinomas) revealed, in addition to *IGF2* and *FGFR1*, 89 other genes that displayed at least threefold differential expression between adrenocortical carcinomas and the normal cortex, and the adrenocortical adenomas ($P < 0.01$). Included in these genes were, along with those already known to be upregulated in adrenocortical tumors such as *IGF2*, *FGFR1*, *TOP2A*, *Ki-67*, also some novel and differentially expressed genes such as osteopontin (*SPP*), serine threonine kinase 15 (*STK15*), angiopoietin 2 (*ANGPT2*), *UBE2C*, and others. A particular gene is worth mentioning: the ectodermal-neural cortex-1 (*ENC1*) gene was overexpressed in carcinomas; interestingly, *ENC1* appears to be upregulated in colorectal carcinoma and was recently identified as a potential target of the β -catenin/T-cell factor complex [51]. Upregulation of *ENC1* transcripts in adrenocortical carcinomas is yet another link to the Wnt signaling pathway.

Finally, very recent microarray study focused on the expression profiles of G protein-coupled receptors between in aldosterone producing adenomas [12]. Although the results indicated greater than 25-fold over normal expression for several genes, including *CD47* antigen receptor, the putative purinergic receptor *FKSG79*, and the cholinergic receptor *CHRM1*, major overexpression was observed for the luteinizing hormone receptor (*LHR*) and the aldosterone synthase genes (*CYP11B2*).

Further analysis of the *CYP11B2* and *LHR* expression levels by means of real-time quantitative PCR in 20 normal adrenals and 18 aldosterone producing adenomas indicated that, as expected, all aldosterone-producing samples expressed levels of the *CYP11B2* gene that were outside the range seen in normal adrenals. In addition, nine of the 18 aldosterone producing adenomas also exhibited elevated levels of *LHR*. To determine whether LH treatment is able to increase expression of *CYP11B2*, transient transfections were performed in H295R adrenocortical cells with expression vectors for LH receptor and reporter constructs for the *CYP11B2* promoter. It was shown that LH is indeed able to mediate *CYP11B2* promoter activity in the presence of LH receptor with a maximal 25-fold induction, indicating that LH receptor levels in the adrenal can drive aldosterone production in the presence of LH. Why aberrant expression of the *LHR* would cause hypercortisolism in some cases and hyperaldosteronism in others is still unclear and further studies are necessary to answer this question.

4. Conclusion

To date, a total of 91 adrenal tissue samples have been profiled by various genome-wide expression studies, investigating thousands of genes and transcripts: one of the most significant findings, consistent with a large body of evidence, both from high-throughput and individual marker studies, is increased *IGF2* expression as a characteristic transcriptional event in the pathogenesis of adrenocortical carcinomas. The significance of this finding is two-fold: not only it identifies *IGF2* as an important marker for adrenocortical cancer, but it also points to its uniqueness since there is really no other such major and consistent signal transduction-related alteration. This suggests that interruption of IGF2-induced signal transduction may lead to a significant therapeutic advance in the treatment of adrenocortical cancer. A second, and equally important discovery is the involvement of the Wnt-signaling pathway in both benign and malignant tumors of the adrenal cortex regardless of their molecular or histologic background, from hyperplasia to malignancy. Finally, the fact that a significant number of novel transcripts were also identified among the most abundant and tissue-specific ones, emphasizes the gaps in current knowledge about adrenocortical cell function and differentiation. We conclude that adrenal expression studies provided reproducible and important data in the search of better understanding of the process of this tissue's differentiation, normal function and tumorigenesis. As this chapter went into press three more relevant studies, including one on the Y1 mouse adrenal cells, were published and largely confirmed the above data [52-54].

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