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Gene expression profiling of human adrenocortical tumors using cDNA microarrays identifies several candidate genes as markers of malignancy

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Abstract:

The aim of this study was to identify predictor sets of genes whose over- or under-expression in human sporadic adrenocortical tumors would help to identify malignant versus benign tumors and to predict post-surgical metastatic recurrence. For this, we analyzed the expression of 230 candidate genes using cDNA microarrays in a series of 57 well characterized human sporadic adrenocortical tumors (33 adenomas and 24 carcinomas). We identified two clusters of genes (the *IGF2* cluster containing 8 genes including *IGF2*, and the Steroidogenesis cluster containing 6 genes encoding steroidogenic enzymes plus 8 other genes) whose combined levels of expression appeared as good predictors of malignancy. This predictive value was as strong as that of the pathological score of Weiss. The analysis of the population of carcinomas (13 tumors) for genes whose expression would be strongly different between recurring and non-recurring tumors allowed the identification of 14 genes meeting these criteria. Among these genes, there are probably new markers of tumor evolution that will deserve further validation on a larger scale. Taken together, these results show that the parallel analysis of the expression levels of a selected group of genes on microgram quantities of tumor RNA (a quantity that can be obtained from fine-needle aspirations) appears as a complementary method to histopathology for the diagnosis and the prognosis of evolution of adrenocortical carcinomas.

Introduction:

Although adrenocortical tumors are highly prevalent in the human population (3 to 7% at autopsy and/or in radiological series), only a small proportion cause endocrine disorders and less than 5% are malignant (1, 2). Many of the tumors that do not secrete aberrant levels of steroids are now discovered as incidentalomas (3). The most frequent clinical presentations of adrenocortical adenomas and carcinomas are Cushing's syndrome (hypersecretion of glucocorticoids) with or without associated androgen secretion and virilism, and Conn's syndrome caused by small adenomas (less than 20 mm in size) exclusively secreting aldosterone. Estrogen-producing tumors are rare and usually malignant. About 50% of adrenocortical carcinomas secrete steroid precursors with reduced bioactivity. On average, adrenocortical carcinomas are diagnosed rather late at a stage when the tumor has often already spread occult or detectable metastases. The prognosis of adrenocortical carcinomas is therefore very poor with a mean patient survival rate of 20% at 5 years (4).

Despite some recent progress, the understanding of the biology of adrenocortical tumors is still partial. Clonal composition analyses and comparative genomic hybridization experiments have established that adrenocortical tumorigenesis is a multistep process resulting from sequential genetic alterations leading to progression from normal to adenomatous and, eventually, to malignant phenotypes (5-8). Several studies have demonstrated an increased frequency of DNA copy number changes in large malignant tumors that are infrequent in small benign lesions. The most common modification, observed in 85% of carcinomas, is the overexpression of the insulin-like growth factor II (*IGF2*) gene, that is associated with paternal isodisomy at the 11p15 locus (9, 10). However, the high number of chromosomal alterations and dysregulations of gene expression that are observed suggests that each tumor is potentially distinct from all others at the molecular and clinical levels (8, 9, 11, 12). Prognosis is thus not likely to be associated with abnormal expression of a single gene but rather with the combined disturbances of several genes. Massively parallel molecular analyses should then be developed in order to identify such combinations of misexpressed genes. The recently developed cDNA array technology, which allows the simultaneous analysis of mRNA expression levels of hundreds of genes, may be the method of choice. Several recent studies have demonstrated that the determination of gene expression signatures among clinically and histologically homogeneous groups of tumors permits the identification of subgroups with different evolution prognosis (13-18).

The classification of adrenocortical tumors between adenomas and carcinomas relies on several criteria including the size of the resected tumor mass, the degree of invasiveness and the

histopathological examination. Tumors presenting with local or regional invasion and/or metastases (McFarlane stages III and IV) are unequivocally classified as malignant. Conversely, in the case of purely localized tumors, the diagnosis is based on histopathological examination using the criteria defined by Weiss (19, 20). However, in ~30% of these tumors (1≤ Weiss score ≤3), histopathology is unable to provide an unambiguous answer. There is therefore a crucial need for additional reliable analyses that could help the clinician in diagnostic and subsequent therapeutic decisions. In a recent retrospective study on a large cohort of patients with sporadic adrenocortical tumors, the analysis of 3 molecular alterations (loss of heterozygosity at the *17p13* locus, uniparental disomy at the *11p15* locus and overexpression of the *IGF2* gene) revealed that they were independent predictors of shorter disease-free survival in univariate analysis (9). In a multivariate analysis, histological grade (relative risk, 4.2) and 17p13 LOH (relative risk, 21.5) appeared to be independently associated with recurrence (9).

The aim of this study was to take advantage of the cDNA array technology to identify predictor sets of genes whose over- or under-expression in adrenocortical tumors would help, on one hand, to discriminate between benign and malignant tumors, and, on the other hand, to identify the subgroup of patients bearing carcinomas at high risk of recurrence, in whom adjuvant therapy may be applied. For this purpose, using cDNA microarrays, we analyzed the expression of 230 candidate genes in a series of 57 sporadic adrenocortical tumors from adult patients who had been carefully followed up by five medical centers within the French clinical network COMETE (COrtico & MEdullo-surrénales Tumeurs Endocrines). We identified two clusters of genes whose levels of expression appeared as good predictors of malignancy. Their combination improved their predictive value to a level similar to but not better than the pathological score of Weiss. In addition, we identified a set of 14 genes (including *ITGB2*, *GZMA*, and *ATF1*) that allowed to significantly predict metastatic recurrence among the 13 carcinomas included in the analysis.

Materials and Methods:

Tumor samples and RNA extraction:

Samples of primary sporadic adrenocortical tumors were obtained from 57 adult patients (14 men, 43 women) referred to and followed by the Endocrine Departments of Cochin, Georges Pompidou, Gustave Roussy, Armand-Trousseau and Albert Michallon Hospitals (Paris, Villejuif and Grenoble, France). We excluded cases with Conn adenomas, i.e. with tumors of 20 mm or less with a pure aldosterone hypersecretion. Written informed consent for germ-line

and somatic DNA analysis was obtained from each patient and the study was formally approved by an institutional review board (CCPPRB Paris-Cochin, July 1996). Access to the collected information was obtained from all of the patients in accordance with national ethic rules. The median age of the patients at the time of diagnosis was 41 years (range 18-79). After surgical resection, tumors were dissected : a fragment was processed for paraffin inclusion and further histopathological examination whereas adjacent pieces were immediately frozen in liquid nitrogen and stored either in liquid nitrogen or at –80 C until RNA extraction and further molecular analysis. Tumors were classified according to Weiss criteria : for each tumor, a Weiss score between 0 and 9 was determined according to the presence or absence of nine predefined histological features (19, 20). The group of tumors analyzed in this study included 33 adenomas (Weiss score $\leq$3) and 24 carcinomas (Weiss score$\geq$4). The major characteristics of the patients included in this study and of their tumors are summarized in Table I.

Total RNA was extracted as previously described (21) and purified by ultracentrifugation through a cesium chloride gradient. The integrity of RNA was confirmed on ethidium bromide-stained gels.

Quantitative RT-PCR:

2 µg of total RNA were reverse-transcribed for 30 min at 37C with SuperScript II RnaseH reverse transcriptase (Invitrogen, Cergy Pontoise, France) under conditions recommended by the manufacturer. Aliquots (1/25 of the RT reaction volume) were subjected to quantitative real time PCR on a Light Cycler™ apparatus (Roche Diagnostics, Meylan, France). The PCR reactions were performed using the following primers : (GCATCGTTGAGGAGTGCTGTTTC and GGGGTATCTGGGGAAGTTGTCC) for IGF2, (TCCACCCACCTGGCTTCAT and GCAGGACCTGGGCTTGTG) for HSD3B1, (GGCAAATGCTTTCGCCTCTGGGTC and TTGTTGGTTTTCGGAACTGAGC) for 18S ribosomal RNA and SYBR green PCR core reagents (LightCycler-FastStart Master SYBR Green I, Roche Diagnostics) according to the manufacturer's instructions. PCR conditions were: step 1: 95 C for 10 min; step 2: 40 cycles consisting of 95 C for 15 s, 57C for 6s (IGF2) or 59C for 5s (HSD3B1 and 18S), and, 72 C for 12 s (IGF2) or for 10s (HSD3B1 and 18S). The samples were analyzed in duplicate and the results were normalized to 18S expression levels.

Preparation of cDNA microarrays:

Gene expression was analyzed by hybridization of arrays with radiolabeled probes. The arrays contained PCR amplification products of 230 human cDNA clones obtained from the IMAGE

consortium through either the RZPD (Berlin, Germany) or the Human Genome Mapping Project Resource Center (Hinxton, UK). These included 187 “cancer-related“ genes (including genes encoding cell cycle control proteins, growth factors, growth factor receptors, transcription factors, cell adhesion molecules, proteins involved in cell invasion, angiogenesis and chemoresistance), 34 “adrenal cortex-specific“ genes (including genes encoding hormone receptors, components of the cAMP signalling pathway, steroidogenic enzymes and components of the IGF2 system) and 9 control genes. Their identity was verified by restriction mapping and/or 5’tag-sequencing of plasmid DNA. The use of control clones, PCR amplification and robotical spotting onto Nytran-N+ membranes (Schleicher & Schuell, Ecquevilly, France) were performed as described in (22). The list of spotted genes is available upon request.

Data analysis and statistical methods:

Hybridization signals (mean values of duplicate spots) were recorded and quantified with a ß-imager (Fuji BAS 5000). The values were then corrected with Image Gauge software for the amount of spotted DNA and the variability of experimental conditions, normalized as a ratio to the total amount of membrane-bound radioactivity, log-transformed and displayed as relative values median-centered in each row and column (14, 22). Average-linkage hierarchical clustering was then applied to determine the closest proximity between tumor samples and between gene expression levels using the Cluster program (with Pearson correlation as similarity metric) and the results were displayed using the TreeView program (23). Genes distinguishing clinical parameters (adenoma vs carcinoma; recurrences) were searched using Student T-test.

Survival analysis was performed using the Kaplan-Meier method (24) and compared between groups using the log rank test (univariate analysis). The calculations were performed using StatView software.

Results:

Gene expression profiling of human adrenocortical tumors:

The mRNAs from 57 human adrenocortical tumor samples were hybridized with cDNA arrays carrying the 230 selected genes. The overall expression patterns of these 57 tumors were analyzed with hierarchical clustering and displayed in a color-coded matrix (Figure 1A). Tumor samples are classified on the horizontal axis and genes on the vertical axis and both are ordered on the basis of similarity of their expression profiles. Overall similarity of gene expression profiles is shown as a dendrogram where branch length is inversely correlated with similarity. As shown in Figure 1B, the two groups of tumors separated on the basis of the expression profiles of all genes contained clearly distinct percentages of adenomas (green squares) versus carcinomas (red squares). Supervised analysis allowed to highlight 2 clusters of genes associated with the adenoma/carcinoma distinction (Figure 1 red curve). They are indicated by colored lines on the dendrogram at left of Figure 1. Statistical analysis of the expression of each gene belonging to these two clusters revealed that they were significantly distinct between adenomas and carcinomas (Figure 1A, right panel). The identity of the genes from each cluster is presented in Table 2. The "*IGF2*" cluster contains 8 genes which encode growth factors (*IGF2*, *TGFB2*), growth factor receptors (*FGFR1*, *FGFR4*, *MST1R*, and *TGFBR1*) as well as *KCNQ1OT1* (also known as *LIT1*, ie long QT internal transcript1) and GAPD, a presupposed housekeeping gene. The "steroidogenesis cluster" contains 14 genes. Six of them encode proteins directly involved in the steroid biosynthesis pathway : *StAR* (steroidogenic acute regulatory protein), *CYP11A* (Cholesterol side chain cleavage enzyme), *HSD3B1* (3ß-hydroxysteroid dehydrogenase/Δ5-4 isomerase type I), *CYP11B1* (steroid 11ß-hydroxylase), *CYP21A2* (steroid 21-hydroxylase), *CYP17* (steroid 17α-hydroxylase). It also contains *PPM1A* (protein phosphatase 1A), *S100B* (S100 calcium-binding protein, ß chain), *GPC3* (glypican 3), *INHA* (inhibin α chain), *CREM* (cAMP response element modulator), *RB1* (Retinoblastoma 1), *NME1* (Non-metastatic protein 23, NM23) and *TGFBR3* (transforming growth factor ß type III receptor).

We then clusterized again the tumors as a function of the expression profiles of each individual gene cluster (Figure 2). The level of expression of the IGF2 cluster allowed to separate a subpopulation (low expression) among which 90% of the tumors were adenomas, from another (high expression) among which 75% were carcinomas. On the opposite, considering the steroidogenesis cluster, low level of expression correlated with carcinomas (81% in the

low-expressing group) whereas high level of expression correlated with adenomas (93% in the high-expressing group).

In order to confirm these results by another quantitative method, we analyzed the mRNA expression level of one representative gene from each cluster, namely IGF2 and HSD3B1, by real-time quantitative RT-PCR. The results shown in Figure 3 indicated a good correlation between both methods.

Disease-free survival analysis of the whole tumor population :

Out of the 57 patients whose tumor RNAs were analyzed on DNA microarrays, 8 had incomplete surgery and 9 had unsufficient follow-up. We could however include 40 patients in a retrospective study of recurrence-free survival. In this group, the median duration of follow-up was 33 months (range 4-97). For patients without recurrence, the minimal follow-up period was 12 months. The Kaplan-Meier estimate of disease-free survival was 82.5% at 2 years (Figure 4A). During the follow-up period, 4 patients died of metastatic recurrence. Seven patients (17.5%) underwent recurrence 2-10.5 months after initial surgery (median= 6.7 months).

Univariate analyses were performed using the Kaplan-Maier statistical method. We first analyzed recurrence-free survival as a function of the expression profile of each individual gene cluster. The results of Figure 2 were used to classify each individual tumor into two groups characterized by low or high expression of the considered cluster. The curves shown in Figure 4 clearly indicate that the IGF2 ($p< 0.01$) and Steroidogenesis ($p< 0.01$) clusters were significant predictors of malignancy. They were not as good predictors however as the pathological grade (Weiss score, Figure 4E) as the rate of recurrence among the group of tumors presenting a Weiss score ≥ 4 was 54% whereas it was only about 40% among the groups that presented either a high level of expression of the IGF2 gene cluster or a low expression level of the Steroidogenesis cluster. However, the combination of these two latter gene clusters allowed the identification of a subpopulation of tumors with low expression of the Steroidogenesis cluster and high expression of the IGF2 cluster that presented the same risk of post-surgical recurrence as the group of tumors with a Weiss score ≥ 4 (Figures 4E and 4F). This observation is the first demonstration for adrenocortical tumors that the analysis of the pattern of expression of a limited number of genes (22 genes in total) is as predictive as the pathological grading.

Analysis of the steroid secretion profiles:

Another question that can be adressed in this study is the relationship between the steroid secretion profiles and the genetic expression profiles of these tumors. Among the tumor population studied, 23 overproduced glucocorticoids, 2 overproduced mineralocorticoids, 17 had a mixed secretion profile and 8 were non-secreting. Three tumors overproduced androgens and 2 overproduced estrogens. As shown in Figure 5, most glucocorticoid-secreting tumors (19 out of 23, 82%) presented a high level of expression of the Steroidogenesis cluster. Unexpectedly however, most (16 out of 19; 84%) of these differentiated tumors presented a low level of expression of the IGF2 cluster. On the other hand, most tumors with a mixed steroid production profile and most non-secreting tumors preferentially expressed the IGF2 cluster at a high level and the steroidogenesis cluster at a low level. This may reflect the known observation that glucorticoid overproduction is observed more frequently in adenomas than in carcinomas (2, 4).

Disease-free survival analysis of adrenocortical carcinomas :

We then separately analyzed the group of carcinomas (Weiss score ≥4) and asked whether we could identify a gene cluster whose expression level could allow to discriminate between recurring and non-recurring tumors. Unfortunately, only 13 of these 24 carcinomas could be included in a retrospective study of recurrence-free survival as tumors that were already metastatic before surgery had to be excluded from the analysis. Supervised analysis using a Student T test at 3% risk lead to the identification of 14 genes whose expression levels were clearly distinct between recurring and non-recurring tumors (Table 3; Figure 6A). These genes were not associated to any particular cluster (Figure 1A, blue curve), probably because of the small number of samples. As shown in Figure 6B, the group of recurring carcinomas could be perfectly separated from the group of non-recurring tumors on the basis of overexpression (6 genes) or underexpression (8 genes) of these genes.

Discussion:

An increasing number of studies have suggested the usefulness of cDNA array-based gene expression profile determination for cancer classification and some of them have succeeded in bringing up novel prognostic information (13-18). Using this technology, we profiled a series of 57 human sporadic adrenocortical tumors using a set of candidate genes, the majority of which are implicated in oncogenesis or steroidogenesis. For 40 of these tumors, the follow-up and clinical information was sufficient to perform a retrospective study. We aimed at identifying groups of genes whose expression level would help, on one hand, to discriminate

between benign and malignant tumors, and, on the other hand, to identify the subpopulation of carcinomas that are more likely to recur.

Two independent clusters of genes (the IGF2 and Steroidogenesis clusters) were independently found to significantly discriminate between two populations of tumors with distinct clinical outcome. Their combination allowed to identify a subpopulation of tumors with both high expression level of the IGF2 cluster and low expression of the Steroidogenesis cluster that have a higher probability of metastatic recurrence over a period of 24 months (60% of this group of tumors recurred). The analysis of the expression level of this group of 22 genes appeared almost as powerful as the well recognized histological score of Weiss at identifying the true carcinomas. Although it may appear disappointing that such a sophisticated genetic analysis using cDNA microarrays does not overperform the routinely used histopathological method in terms of prediction power, one should acknowledge that the possibility to miniaturize this technique opens new fields for the diagnostic analysis of adrenocortical tumors. We show here that the analysis of the expression level of a group of 22 genes is sufficient to predict metastatic recurrence with the same index of confidence as the histological score of Weiss. Since 1 µg of total tumor RNA is certainly sufficient to carry out this genetic analysis (2 µg of RNA were used in the present study), these results open the way to the molecular diagnostic performed before the surgery on a needle biopsy of the tumor. As recently reviewed, fine-needle aspirations appear as a sensitive and accurate method to collect biological material for the diagnosis of adrenocortical masses (25).

The identity of the genes that cosegregate with *IGF2* in the so-called IGF2 cluster is extremely interesting. Two genes encode fibroblast growth factor receptors of the FGF family : *FGFR1* and *FGFR4*. The fibroblast growth factors FGF-1 and FGF-2 are expressed in the adrenal cortex. They are the most powerful mitogens for adult steroidogenic adrenocortical cells (26) as well as for the human adrenocortical tumor cell line NCI-H295R (27). They are also known to stimulate the proliferation of endothelial and mesenchymal cells. They bind to a family of four tyrosine kinase receptors, among which FGFR1 and FGFR4 are the most strongly expressed in the adrenal cortex (28, 29). The overexpression of these two FGF receptors in adrenocortical cancers is thus likely to participate in their increased proliferation and vascularization. Then, FGF receptors potentially represent new targets for novel therapeutic approaches of adrenocortical carcinoma, a type of cancer that turns out to be resistant to conventional antimitotic agents (30). Two genes encode TGFß2, a member of the TGFß superfamily and its signalling serine/threonine kinase receptor TGFß-R1. TGFß1 is an autocrine factor produced

by the adrenal cortex (31, 32). It has no effect on the proliferation of steroidogenic cells but it strongly inhibits their steroidogenic activities through down-regulation of StAR and CYP17 expression (33, 34). Although TGFß2 has been less characterized in this tissue, it is known to share the same type 1 and type 2 receptors as TGFß1, to signal through the phosphorylation of the same Smad proteins (Smad2 and Smad3) and to have similar inhibitory effects on adrenocortical steroidogenesis (our unpublished observations). It is tempting to speculate that the overexpression of TGFß2 and its type 1 receptor in adrenocortical carcinomas may participate in the dedifferentiation of these tumors. Indeed, in our study, 6 out of 8 non-secreting tumors presented with a high level of expression of the IGF2 cluster (Figure 6). Two previous studies have analyzed TGFß1 expression in adrenocortical tumors and have characterized a decreased expression of both the protein and the mRNA in carcinomas as compared to normal tissue and adenomas (35, 36). In our study, both TGFß1 and TGFß2 cDNA probes were present on the arrays, but only TGFß2 appeared to cosegregate with other genes and to be overexpressed in carcinomas. Complementary experiments are required to establish whether there is a shift in expression from TGFß1 toward TGFß2 during adrenocortical tumor progression. One gene (*MST1R*) encodes Ron, a tyrosine kinase receptor for macrophage stimulating protein-1 that has been reported to be expressed in the adrenal glands, both at the embryonic and the adult stages (37). In adrenomedullary PC12 cells, macrophage stimulating protein 1 has been shown to be a potent mitogen. It is difficult however to speculate about its possible effect in adrenocortical tumors until some experiments are carried out in this cell system. Another gene of this cluster is *KCNQ1OT1*, which belongs to the same 11p15.5 region as the *IGF2* gene. *KCNQ1OT1* encodes a non-coding antisense transcript within intron 10 of the *KCNQ1* gene and might be involved in the regulation of parental imprinting of the centromeric domain of the 11p15 region. The *KCNQ1OT1* and the *IGF2* genes are the only known genes in the 11p15 region that are maternally-imprinted and paternally-expressed. We previously showed that the main mechanism for overexpression of the *IGF2* gene in malignant adrenocortical tumors was a paternal isodisomy (loss of the maternal allele and duplication of the paternal allele) (10, 21). It is therefore not surprising to find the concomittant expression of these two genes in the *IGF2* cluster. The last gene belonging to this cluster is *GAPD*, encoding glyceraldehyde-3-phosphate dehydrogenase, a presupposed housekeeping gene. It has been reported previously that *GAPD* is up-regulated under a variety of circumstances including hypoxia and apoptosis (38, 39), two events that are likely to occur in the center of solid tumors. It is therefore not so unexpected to find this gene overexpressed in the population of adrenocortical carcinomas. It should be noted that this overexpression could not result from a

technological artefact since *GAPD* overexpression would be unlikely to non-randomly occur in one particular subpopulation of tumors.

Out of the 14 genes present in the *Steroidogenesis* cluster, 6 encode enzymes or proteins directly involved the steroid biosynthesis pathway: the mitochondrial protein StAR, that facilitates the tranfer of cholesterol to the inner mitochondrial membrane, the cytochromes P450scc (cholesterol desmolase), P450c17 (17α-hydroxylase), P450c21 (21-hydroxylase), P450c11B1 (11ß-hydroxylase) and 3ß-hydroxysteroid dehydrogenase-isomerase. However, the correlation between the level of expression of these different enzymes in each individual tumor and their steroid secretion profile is not trivial. This indicates that the level of expression of some regulatory proteins, acting at the post-transcriptional level to modulate protein expression and/or enzymatic activity, has to be taken into consideration to fully explain the steroidogenic profiles of the tumors.

Another original observation in this study is the identification of a group of 14 genes whose expression level allowed to perfectly discriminate recurring from non-recurring tumors among the 13 carcinomas analyzed. Among these genes is *ITGB2*, encoding integrin ß2, a common subunit for several receptors specifically expressed at the surface of leukocytes (40). Integrin ß2 associates with distinct subunits to form αLß2, αMß2, αDß2 or αXß2 integrins. These integrins are receptors for ICAM1-4 and fibrinogen. Integrin αLß2 (LFA-1) plays an important role in the rolling of leukocytes at the surface of the endothelium and their extravasation, as a receptor for ICAM-1 and ICAM-2 (41). This increased expression of *ITGB2* is therefore likely to reflect an increased tumor infiltration by leukocytes. Granzyme A, which is encoded by a gene belonging to this same cluster, is a trypsin-like serine protease that is secretd by T lymphocytes and participates in target cell lysis during cell-mediated immune response (42, 43). Expression of *GZMA*, like that of *ITGB2*, is thus likely to reflect infiltration by immune cells. The inflammatory status of the carcinoma at time of resection could thus represent an important parameter to take in consideration for the prediction of metastatic evolution. The third gene in this cluster that is expressed with markedly distinct intensities between recurring and non-recurring carcinomas is *ATF1*. This gene encodes a cAMP-dependent transcription factor of the CREB family that is ubiquitously expressed. It was recently shown to be expressed in the human tumor cell line NCI-H295R where, together with CREMτ, it functionally compensates for the lack of CREB (44).

In conclusion, this is the first study reporting the parallel analysis of the expression profiles of hundreds of genes in human sporadic adrenocortical tumors in correlation with the clinical follow-up of the cancer patients. The deliberate selection of a limited number of known genes with established biological functions (230 in this study) prevents us from extrapolating too much about the etiology of the disease. Certainly, a pangenomic analysis may be more successful in identifying new genes associated with adrenocortical tumorigenicity. During the course of the preparation of this manuscript, two such studies appeared in the literature (45, 46). Giordano et al. analyzed the expression of 10,500 unique genes in a series of 11 adrenocortical carcinomas and 4 adenomas and compared these expression profiles with that of normal adrenal cortex tissue (45). However, no prognostic analysis based on clinical follow-up was carried out in this extensive work. Interestingly, several of the most differentially expressed genes identified in this large scale study were also found in our clusters, including IGF2, FGFR1 and CYP11B1. In the most recent study, Bourdeau et al. analyzed the expression of a similar number of genes in a series of 8 ACTH-independent macronodular adrenal hyperplasia and identified candidate genes up- and down-regulated in this rare disorder (46). The approach that we used has proven successful in identifying a set of 22 predictor genes which are altogether reasonably good indicators of malignancy. It has also identified a cluster of 14 genes that seems to have a prognostic interest to identify the subpopulation of carcinomas that are at risk of post-surgical recurrence. Given the small size of this latter group of carcinomas analyzed, these results will deserve further validation on a larger population and through the use of complementary techniques such as in situ hybridization or immunohistochemistry.

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Legends to the Figures:

Figure 1: Expression patterns of 230 genes in 57 human sporadic adrenocortical tumors.

A- Each row represents a single gene and each column represents a single tumor sample. Genes are referenced by their HUGO abbreviation as used in Locus Link (http://www.ncbi.nlm.nih.gov/LocusLink/). The results are shown as relative expression levels (relative to the median value of each row and each column) and are represented with a color scale indicated at the bottom ranging from 1/100 to 100-fold changes. Red and green indicate expression levels respectively above and below the median. The clustering program arranges samples along both vertical and horizontal axis so that the most similar profiles are placed adjacent to each other. The length of the branches of the dendrograms capturing respectively the tumor samples (top) and the genes (left) reflects the similarity of the related elements. Colored dendrogram lines indicate pertinent gene clusters. The curves on the right side of the picture represents genes discriminating carcinoma from adenoma (red curve) and post-surgical recurrences in carcinomas (blue curve).The logarithm (base 10) of p-value of Student T test is plotted. The curves were smoothed with a windows of 5.

B- Extended representation of the list of tumors analyzed in Figure 1A. Each tumor is identified by a number, and classified using either a green (adenoma) or a red (carcinoma) square and either a black (post-surgical recurrence), a yellow (no recurrence) or a white (not included in the follow-up study) square.

Figure 2: Classification of adrenocortical tumors using specific gene clusters.

The 57 adrenocortical tumors were reclustered according to the expression patterns of the two clusters of genes identified in Figure 1A. The percentage of adenomas or carcinomas present within each subgroup resulting from this reclustering is indicated beneath each analysis. The abbreviated names of the genes is indicated in front of each row.

Figure 3: Comparative quantitation of IGF2 and HSD3B1 gene expression in 16 adrenocortical tumors by two distinct methods.

IGF2 and HSD3B1 mRNA levels were determined in 8 carcinomas and 8 adenomas by cDNA arrays (A) or quantitative RT-PCR (B) as described in Material and Methods.

Figure 4: Kaplan-Maier recurrence-free survival analysis in 40 patients with adrenocortical tumor.

(A) : overall recurrence-free survival ; (B) : Recurrence-free survival according to the level of expression of the IGF2 gene cluster ; (C) : Recurrence-free survival according to the level of expression of the Steroidogenesis gene cluster ; (D) : Recurrence-free survival according to the combined levels of expression of the steroidogenesis and IGF2 gene clusters. (E) Recurrence-free survival according to histological grade (Weiss score). The number of samples in each group (n) is indicated over each curve. The probability of significant difference between the survival rates of two groups was analyzed using the Wilcoxon test and is indicated in the lower right corner of each analysis. It could not be calculated when one group contained no recurrence .

Figure 5: Correlation between the steroid secretion profiles and the gene expression profiles of adrenocortical tumors.

Out of the tumor population analyzed in this study, 23 secreted glucocorticoids, 8 were non-secreting and 17 had a mixed secretion profile (glucocorticoids and/or mineralocorticoids and/or androgens). The picture shows the distribution of these three types of tumors among the four groups of gene expression profiles constituted by high or low levels of expression of the IGF2 (IGF) and Steroidogenesis (St.) gene clusters.

Figure 6: Identification of a gene cluster that discriminates recurring and non-recurring carcinomas.

(A) Statistical analysis of the expression levels of each individual gene in recurring and non-recurring carcinomas (13 samples) allowed to identified this group of 14 genes. These tumors were then reclustered according to the expression pattern of these genes. The data are represented as described in Legend to Figure 1. Two distinct subgroups were clearly identified. Black squares correspond to tumors that recurred after surgical removal and yellow squares to non-recurring tumors. (B) : Kaplan-Maier recurrence-free survival analysis of 13 patients with adrenocortical carcinoma and sufficient follow-up according to the separation shown in Figure 6A. The number of samples in each group (n) is indicated near each curve. The probability of significant difference between the survival rates of two groups was analyzed using the Wilcoxon test and is indicated in the lower right corner.

Clinical characteristics	
Pathological tumor size (cm) (min-max; median)	**3 - 30; 8**
Pathological tumor weight (g) (min-max; median)	**8 - 5000; 303**
Tumor extension (n)	
localized	**46**
regional	**2**
metastatic	**9**
Steroid secretion profile (n)	
glucocorticoids	**23**
mineralocorticoids	**2**
androgens	**3**
estrogens	**2**
mixed	**17**
non-secreting	**8**
non described	**2**
Follow-up (months) (min-max; median)	**2.1 - 97; 31**
Relapse-free survival (months) (min-max; median)	**2 - 97; 29**
Death (n)	**8**
Histological grade (n)	
adenoma (Weiss criteria < 4)	**33**
carcinoma (Weiss criteria ≥ 4)	**24**

Table 1: Clinical characteristics of the patients

Gene symbol	Gene/protein identity	Chromosome location
IGF2 Cluster		
IGF2	Insulin-like growth factor-2; somatomedin-A	11p15.5
FGFR1	Fibroblast growth factor receptor-1	8p11.2-p11.1
FGFR4	Fibroblast growth factor receptor-4	5q35.1-qter
TGFB2	Transforming growth factor-ß2	1q41
TGFBR1	Transforming growth factor-ß receptor type I; activin receptor-like kinase 5; ALK5	9q33-q34
MST1R	Macrophage stimulating protein-1 receptor; Ron protein tyrosine kinase	3p21.3
KCNQ1OT1	KCNQ1 overlapping transcript 1; lit1	11p15.5
GAPD	Glyceraldehyde 3-phosphate dehydrogenase	12p13.31-p13.1
Steroidogenesis Cluster		
CYP11A	Cytochrome P450 scc; Cholesterol side chain cleavage enzyme	15q24.1
CYP11B1	Steroid 11ß-hydroxylase; Cytochrome P450 11ß	8q21
CYP17	Steroid 17α-hydroxylase; Cytochrome P450 17α	10q24.3
CYP21A2	Cytochrome P450 c21; steroid 21-hydroxylase	6p21.1
HSD3B1	3ß-hydroxysteroid dehydrogenase/Delta 5 isomerase type 1	1q21.2
STAR	Steroidogenic acute regulatory protein	8p11.2
INHA	Inhibin α subunit	2q33-q36
CREM	cAMP responsive element modulator	10p12.1-p11.2
RB1	Retinoblastoma protein-1	13q14.1-q14.2
PPM1A	Protein phosphatase 1A, magnesium-dependent; protein phosphatase 2Cα	11q23.1
NME1	Non metastatic protein 23 (NM23); Nucleoside diphosphate kinase-A	17q21.3
S100B	S100 calcium binding protein B	21q22.3
TGFBR3	Transforming growth factor ß receptor III; Betaglycan	1p22.1
GPC3	glypican 3	Xq26

Table 2 : Identity of the gene clusters discriminating adenomas from carcinomas

Gene symbol	Gene/protein identity	Chromosome location
ISGF3G	Interferon-stimulated transcription factor 3, gamma	14q11.2
IL2RG	Interleukin 2 receptor, gamma	Xq13
GZMA	Granzyme A	5q11-q12
PTPN2	protein tyrosine phosphatase, non-receptor type 2	18p11.3-p11.2
ITGB2	Integrin ß2 ; macrophage antigen-1 (mac-1)	21q22.3
ATF1	Activating transcription factor 1	12q13
GAPD	Glyceraldehyde 3-phosphate dehydrogenase	12p13.31-p13.1
ACTG1	Actin gamma 1	17q25.3
TLN1	Talin 1	9p
PRKCSH	Protein kinase C substrate 80K-H	19p13.2-p13.1
VIL2	Villin 2; ezrin	6q25-q26
ECE1	Endothelin converting enzyme 1	1p36.1
CDKN2A	Cyclin-dependent kinase inhibitor 2A ; p16	9p21
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	14q24.3

Table 3 : Identity of the set of genes discriminating recurring from non-recurring carcinomas

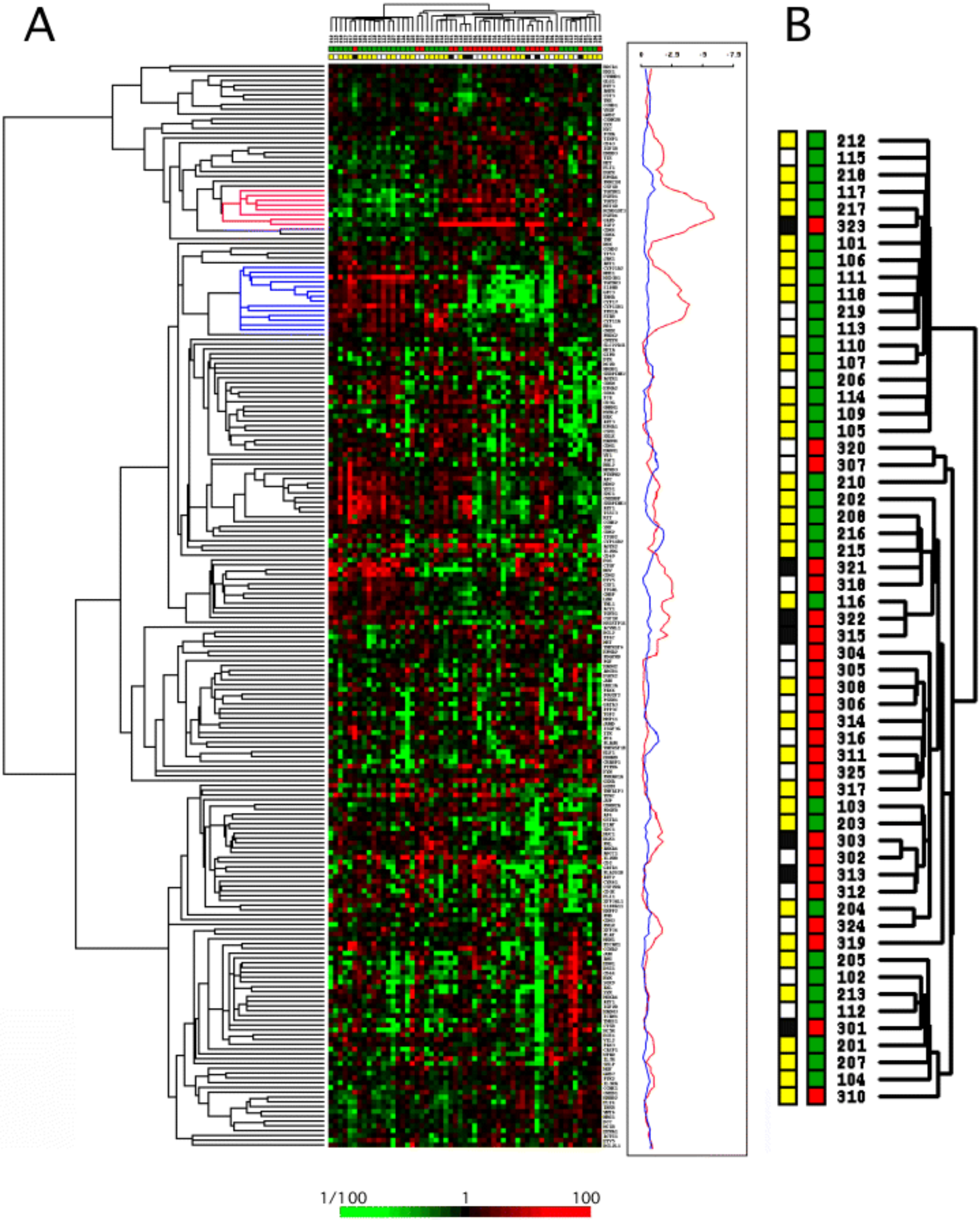
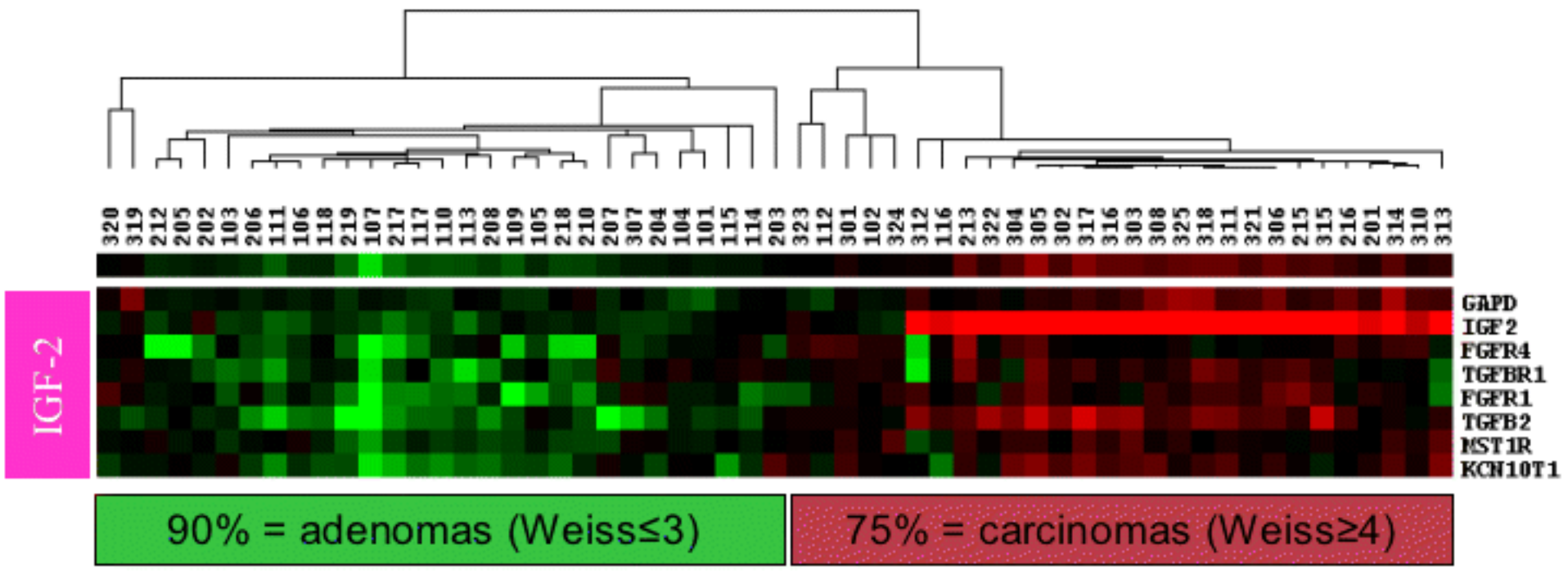

A
B
212
115
218
117
217
323
101
106
111
118
219
113
110
107
206
114
109
105
320
307
210
202
208
216
215
321
318
116
322
315
304
305
308
306
314
316
311
325
317
103
203
303
302
313
312
204
324
319
205
102
213
112
301
201
207
104
310
1/100
1
100

Figure 1


320 319 212 205 202 103 206 111 106 118 219 107 217 117 110 113 208 109 105 218 210 207 307 204 104 101 115 114 203 323 112 301 102 324 312 116 213 322 304 305 302 317 316 303 308 325 318 311 321 306 215 315 216 201 314 310 313
IGF-2
GAPD
IGF2
FGFR4
TGFBR1
FGFR1
TGFB2
MST1R
KCNJ10T1
90% = adenomas (Weiss≤3)
75% = carcinomas (Weiss≥4)

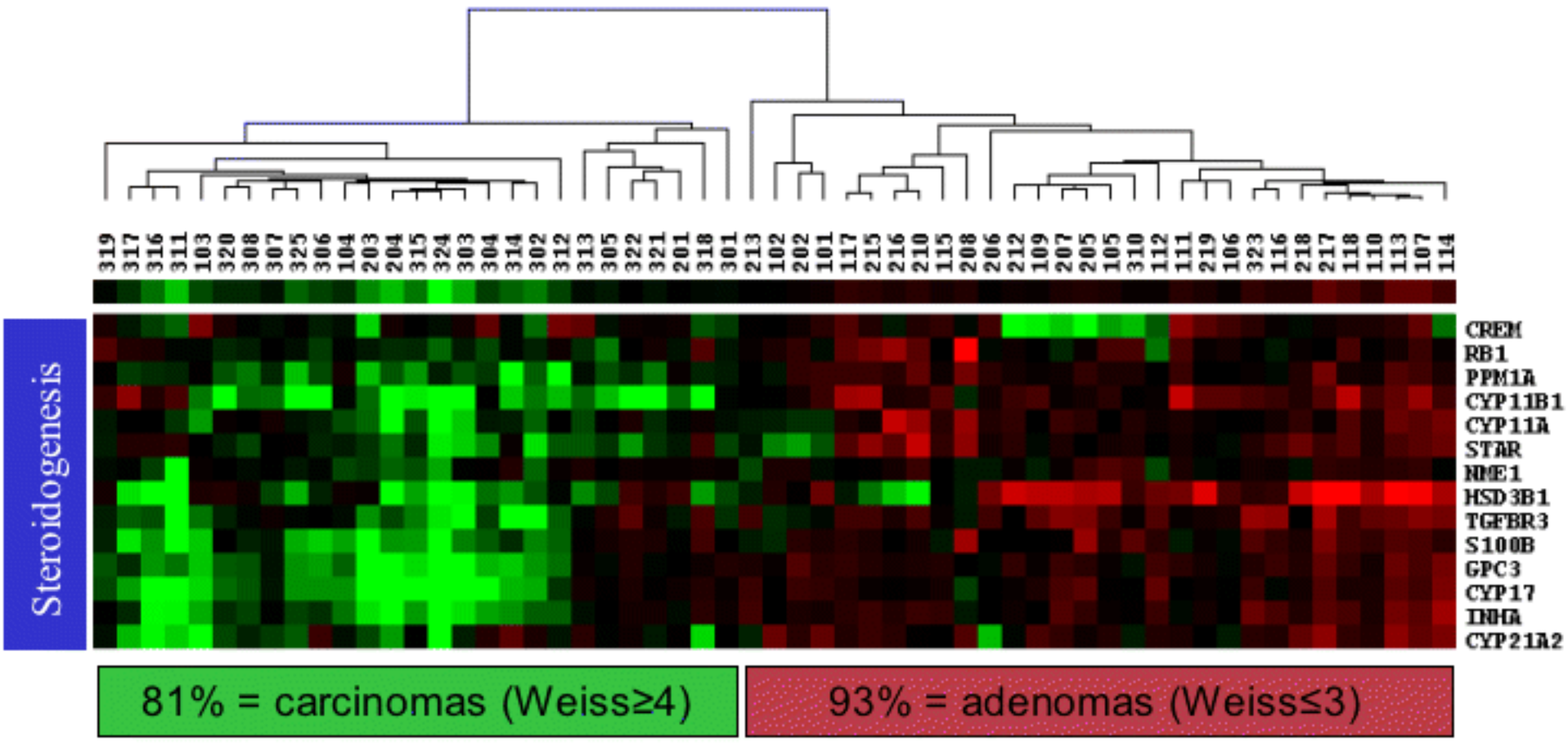
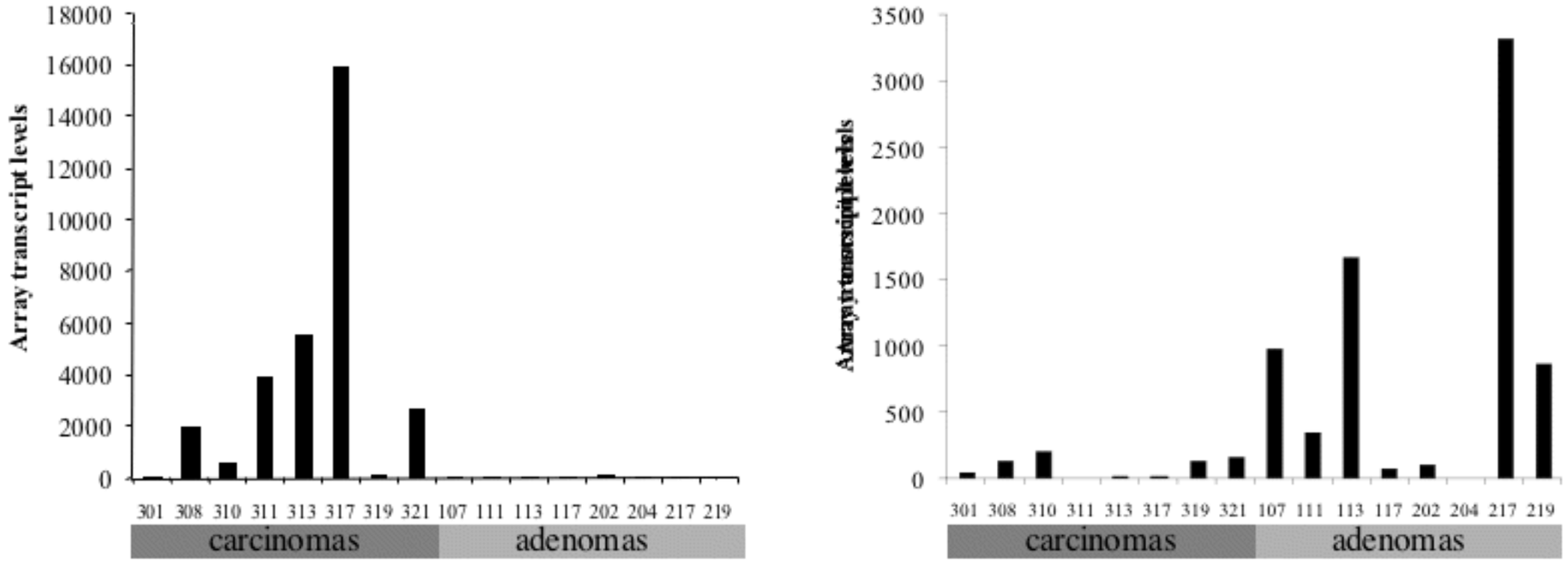

319 317 316 311 103 320 308 307 325 306 104 203 204 315 324 303 304 314 302 312 313 305 322 321 201 318 301 213 102 202 101 117 215 216 210 115 208 206 212 109 207 205 105 310 112 111 219 106 323 116 218 217 118 110 113 107 114
Steroidogenesis
CREM
RB1
PPM1A
CYP11B1
CYP11A
STAR
NME1
HSD3B1
TGFBR3
S100B
GPC3
CYP17
INHA
CYP21A2
81% = carcinomas (Weiss≥4)
93% = adenomas (Weiss≤3)

Figure 2

A


IGF2
HSD3B1
Array transcript levels
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
3500
3000
2500
2000
1500
1000
500
301 308 310 311 313 317 319 321 107 111 113 117 202 204 217 219
carcinomas
adenomas

B

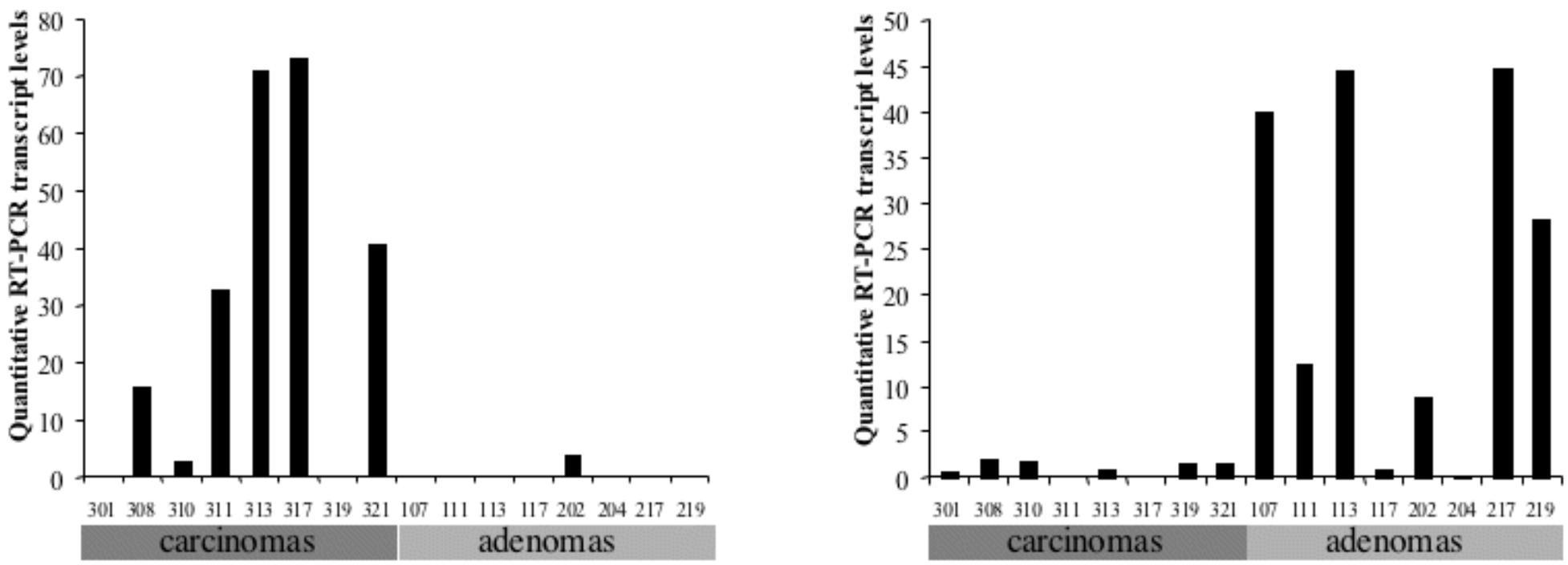

Quantitative RT-PCR transcript levels
80
70
60
50
40
30
20
10
0
45
35
25
15
5
301 308 310 311 313 317 319 321 107 111 113 117 202 204 217 219
carcinomas
adenomas

Figure 3

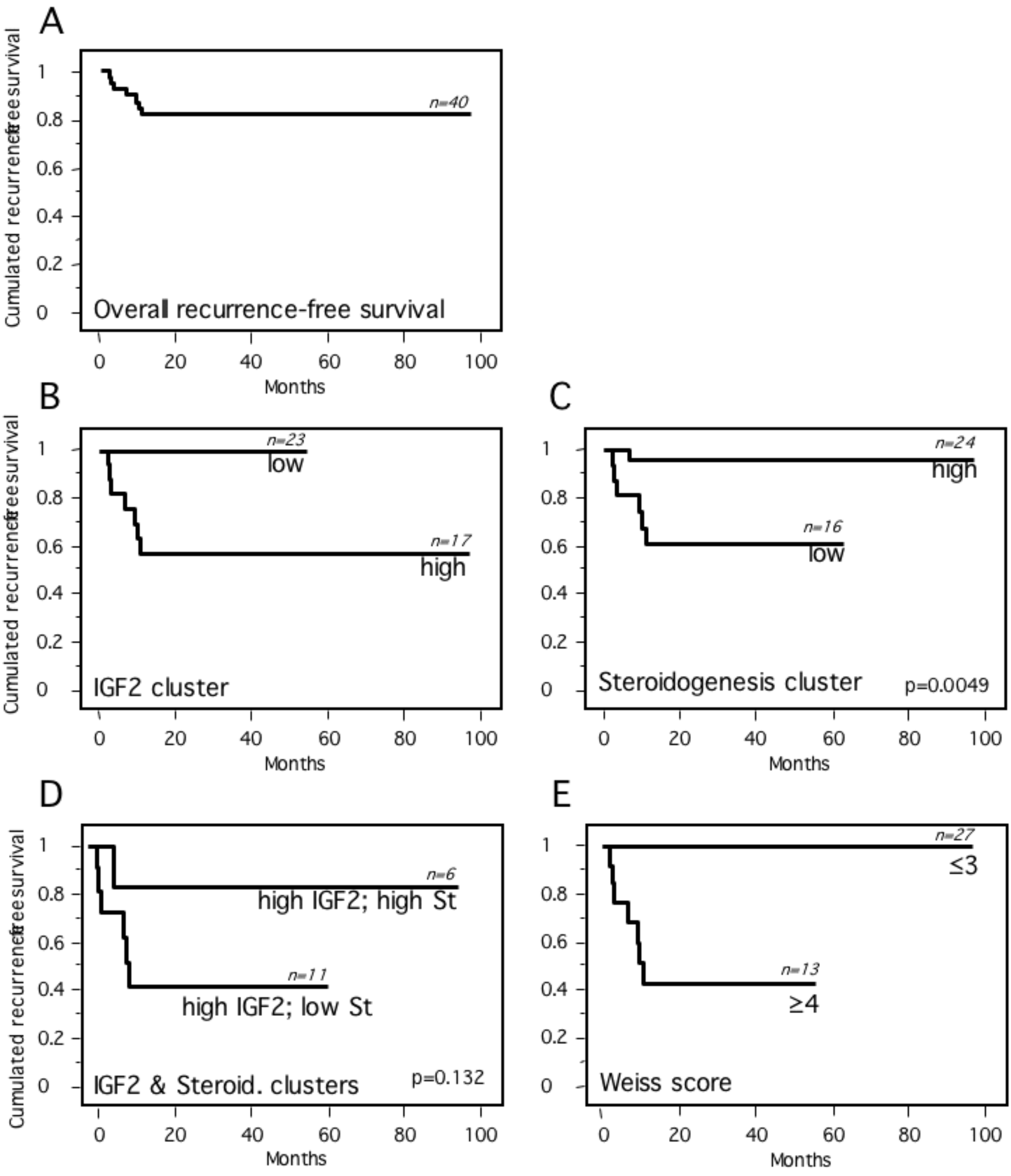

A
Cumulated recurrence-free survival
1
0.8
0.6
0.4
0.2
0
n=40
Overal recurrence-free survival
0
20
40
60
80
100
Months
B
Cumulated recurrence-free survival
n=23
low
n=17
high
IGF2 cluster
Months
C
n=24
high
n=16
low
Steroidogenesis cluster
p=0.0049
Months
D
Cumulated recurrence-free survival
n=6
high IGF2; high St
n=11
high IGF2; low St
IGF2 & Steroid. clusters
p=0.132
Months
E
n=27
≤3
n=13
≥4
Weiss score
Months

Figure 4

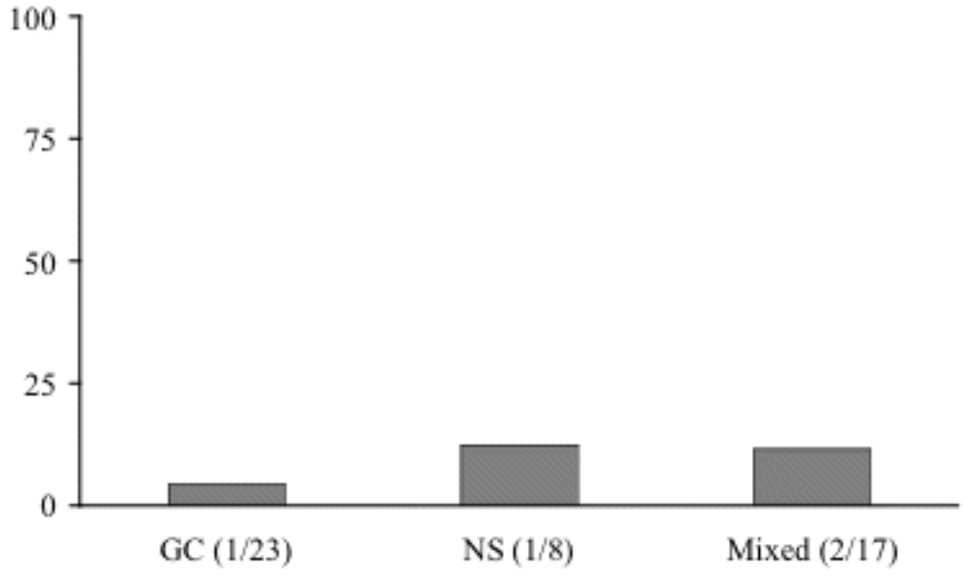
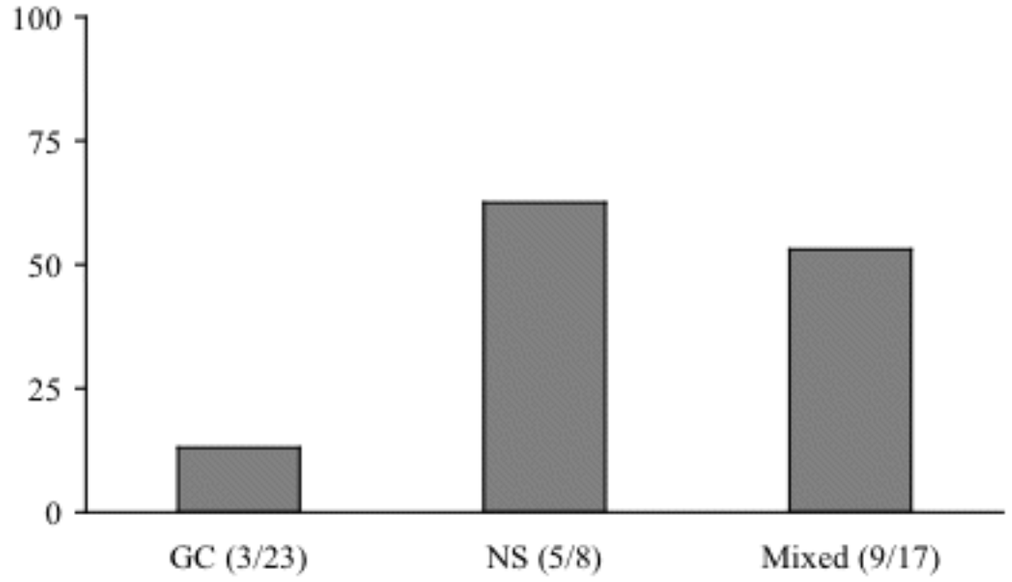
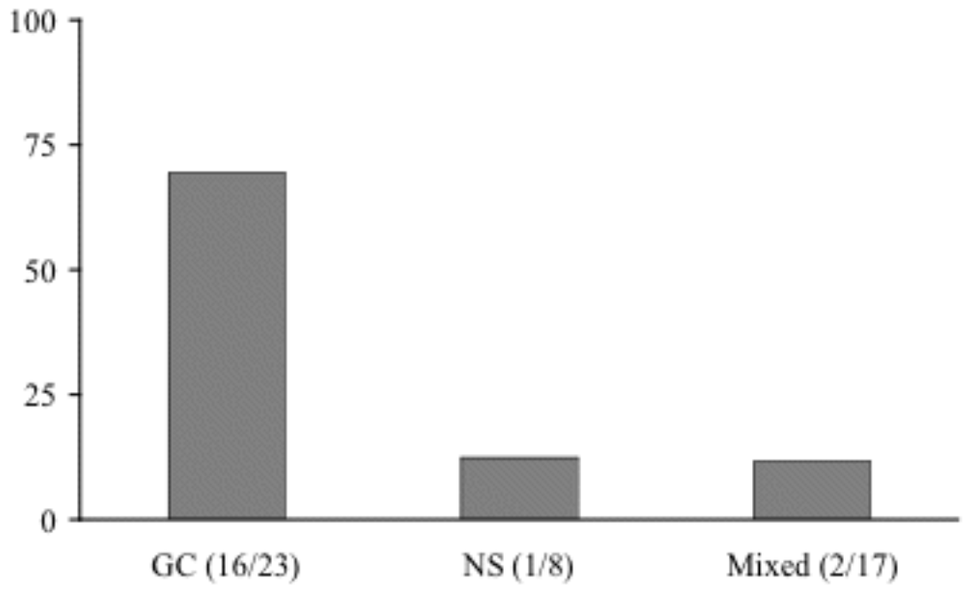
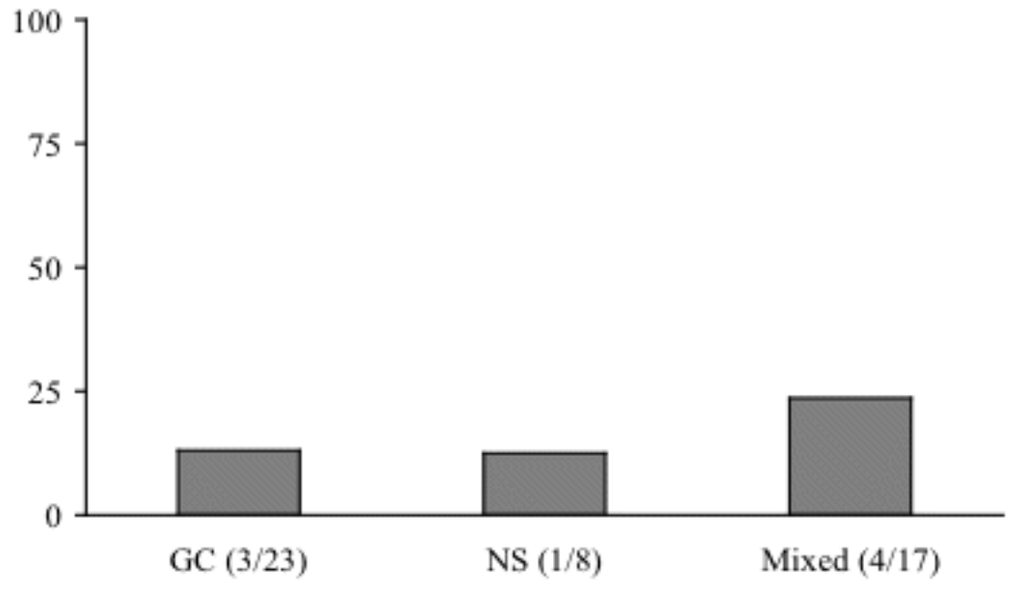
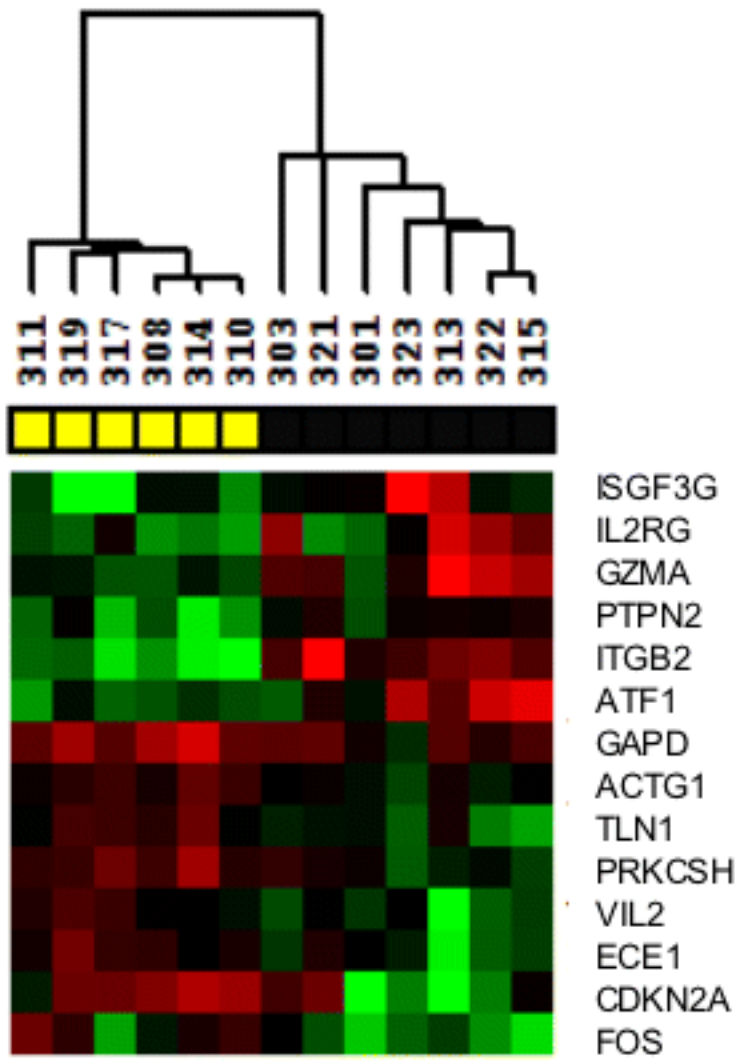
	IGF low	IGF high
St. low		
St. high		

Figure 5

A


311
319
317
308
314
310
303
321
301
323
313
322
315
ISGF3G
IL2RG
GZMA
PTPN2
ITGB2
ATF1
GAPD
ACTG1
TLN1
PRKCSH
VIL2
ECE1
CDKN2A
FOS

B

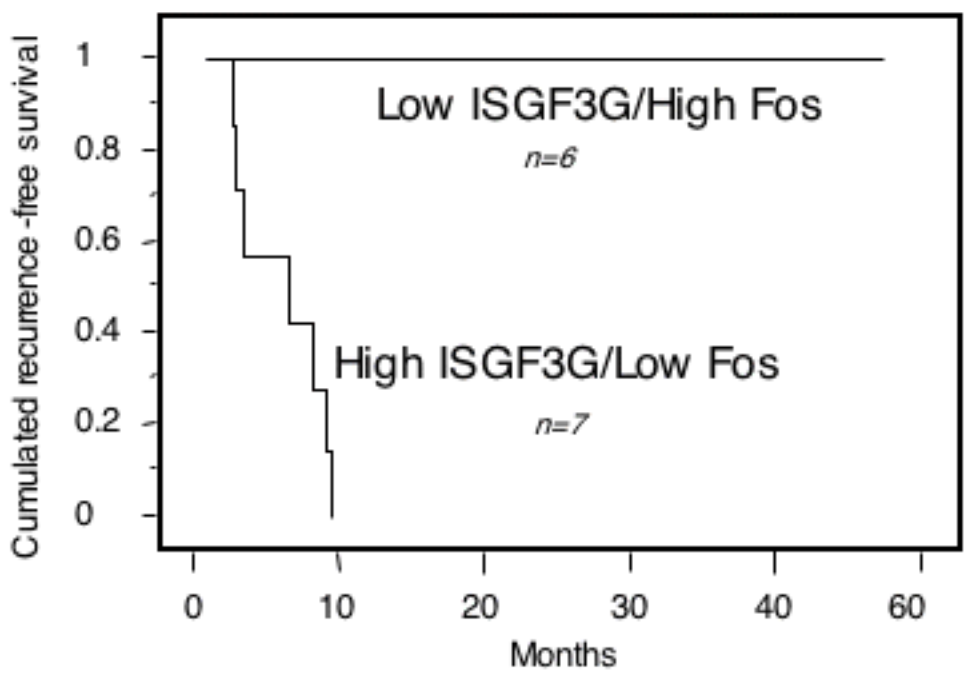

Cumulated recurrence -free survival
1
0.8
0.6
0.4
0.2
0
Low ISGF3G/High Fos
n=6
High ISGF3G/Low Fos
n=7
0
10
20
30
40
60
Months

Figure 6