

Heterogeneity of Aldosterone-Producing Adenomas Revealed by a Whole Transcriptome Analysis

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Abstract—Aldosterone-producing adenomas (APAs) are a common cause of arterial hypertension, but the underlying molecular mechanisms are unknown, although a transcriptional modulation of aldosterone synthase (CYP11B2) has been suggested. Aldosterone synthesis involves 2 main rate-limiting steps: cholesterol transport into mitochondria and CYP11B2 gene transcription. Evidence supports a role of Ca^{2+} /calmodulin-dependent protein kinases (CAMKs) in the regulation of angiotensin II- and potassium-stimulated aldosterone production. CAMK-I mediates CYP11B2 transcription via cAMP response element binding protein and activating transcription factor 1 transcription factors and nuclear receptor Nur-related factor 1. CAMK-II affects cholesterol transport into mitochondria by acting on steroidogenic acute regulatory protein and/or cytoskeleton proteins. We analyzed the whole transcriptome of APAs as compared with a pool of normal human adrenocortical tissues. Based on steroidogenic enzyme gene expression profiles, we identified 2 APA subgroups: 1 featuring overexpression of CYP11B2, CAMK-I, 11- β -hydroxylase, 3- β -hydroxysteroid dehydrogenase, and 21-hydroxylase and the underexpression of CAMK-II and the other one with an opposite profile. The low CYP11B2 group exhibited a longer known duration of hypertension and a lower rate of long-term cure. Thus, aldosterone overproduction in APAs involves complex alterations of aldosterone synthesis regulation rather than simply increased aldosterone synthase gene expression. Whether the molecular signature of APA carries prognostic information is worth further investigation. (*Hypertension*. 2007;50:1106-1113.)

Key Words: secondary hypertension ■ gene expression profiling ■ aldosterone ■ aldosteronism ■ tumors

The Primary Aldosteronism Prevalence in Italy Study, a large prospective survey of consecutive newly diagnosed hypertensive patients referred to specialized hypertension centers, showed that aldosterone-producing adenoma (APA) and idiopathic hyperaldosteronism occur in 4.8% and 6.4% of the hypertensive subjects, respectively.¹ Thus, with a prevalence rate of 11.2%, primary aldosteronism is the most common curable endocrine cause of arterial hypertension.² Notwithstanding this fact, the molecular mechanisms causing autonomous aldosterone hypersecretion in APA are unknown.

Aldosterone-secreting cells store the steroid in minimal quantities and, therefore, the aldosterone secretion is finely regulated and tightly coupled to its biosynthesis. Signaling pathways that are activated by angiotensin II and potassium, via intracellular Ca^{2+} -mediated mechanisms, and by adrenocorticotropin (corticotropin), via adenylate cyclase and cAMP, modulate this process by affecting the 2 main rate-limiting steps of steroidogenesis: the transport of cholesterol in mitochondria (fast regulation) and the transcription of aldosterone synthase gene (CYP11B2; slow regulation).³

A transcriptional modulation of aldosterone overproduction has been suggested based on data showing that the CYP11B2 gene expression would be higher in APA than in normal adrenocortical tissues.^{4,5} Because this modulation depends on *cis*- and *trans*-regulatory elements,⁶ the initial alteration triggering aldosterone excess would reside upstream of CYP11B2, likely at the level of the regulatory elements. Conserved *cis*-elements, including the nerve growth factor-induced clone B (NGFIB) response element (NBRE-1), the Ad5, and the cAMP response element, were mapped in the 5'-flanking region of the CYP11B2 gene. Binding of the transcription factors NGFIB, activating transcription factor 1 (ATF-1), cAMP response element binding protein, and Nur-related factor (NURR1), the activity of which is partially regulated by their phosphorylation state, to these sites turns on the CYP11B2 gene transcription.⁷

The Ca^{2+} /calmodulin-dependent protein kinase (CAMK) family includes 6 members, which are involved in the regulation of angiotensin II- and potassium-stimulated aldosterone production⁸⁻¹⁰ and are activated by binding of the Ca^{2+} /calmodulin (CaM) complex to a CaM-binding domain

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Table. Clinical Features of the Patients With APAs

Variable	Preadrenalectomy	Postadrenalectomy	P
Age, mean±SD, y	50±12
Gender (male/female), %	4/12 (25/75)
Systolic blood pressure, mean±SD, mm Hg	167±13	135±17	<0.001
Diastolic blood pressure, mean±SD, mm Hg	103±10	82±5	<0.001
Serum potassium levels, mean (range), mmol/L	2.9 (0.6)	4.2 (0.6)	0.007
Supine plasma aldosterone levels, mean (range), ng * dL ⁻¹	24.9 (3.0 to 103.0)	7.1 (1.8 to 15.8)	0.039
Supine PRA, mean (range), ng * mL ⁻¹ * h ⁻¹	0.28 (0.10 to 1.90)	1.53 (0.85 to 5.24)	n.s.
ARR, mean±SD, ng * dL ⁻¹ /ng * mL ⁻¹ * h ⁻¹	131±62	10±8	n.s.
Tumor size, mean (range), mm	23 (10 to 75)

PRA indicates plasma renin activity; ARR, aldosterone renin ratio; n.s., not significant; ..., no data.

in response to an increase of intracellular Ca²⁺ concentration.¹¹ CaMK-I is expressed in the adrenal cortex and in the H295R adrenocortical carcinoma-derived cell line.⁸ Moreover, CaMK-I plays a pivotal role in the activation of CYP11B2 transcription by phosphorylating cAMP response element binding protein (CREB), ATF-1, and nuclear receptor NURR1.^{10,11} The regulation of aldosterone production also involves CAMK-II, which acts on cholesterol transport into the mitochondria,¹⁰ a CaM-dependent process that may involve the cytoskeleton proteins.¹² This step requires the activity of steroidogenic acute regulatory protein (StAR), which delivers cholesterol to the inner mitochondrial membrane, where it is converted into pregnenolone by cytochrome P450 side chain cleavage enzyme (P450_{scc}). Whether the excess aldosterone in APA depends on aberrant STAR gene expression and/or upregulated activity by posttranscriptional mechanisms, as phosphorylation of regulatory residues, is unclear, because data in APA are limited and conflicting.^{13,14} Hence, given the complexity of these molecular pathways, we used a whole transcriptome analysis approach to gain insight into the mechanisms causing aldosterone excess in APA.

Methods

Adrenal Specimens and Diagnostic Criteria

Adrenocortical tissues from 16 patients with an APA were studied. Histologically normal adrenocortical tissue obtained at surgery from patients with renal cancer undergoing unilateral nephrectomy and ipsilateral adrenalectomy (n=5) was used as control after the subcapsular region was carefully dissected under a microscope. Dissection of the zona glomerulosa was confirmed by histological examination of hematoxylin/eosin-stained remnant adrenocortical tissue. Moreover, previous experiments confirmed that dissected cells were mostly deriving from zona glomerulosa, with only ≈5% from zona fasciculata. Tissue specimens were obtained at surgery under sterile conditions, immediately frozen in liquid nitrogen, and stored at -80°C until extraction, as described.¹⁵

The diagnosis of APA was based on strict predefined criteria composed of lateralization of aldosterone secretion at adrenal vein sampling, evidence of an adenoma at surgery and pathology, and, more importantly, the follow-up data. For the latter, demonstration of normokalemia and cure or improvement of hypertension ≥120 days after adrenalectomy was required.¹ Cure was defined as normotension without medications and improvement as a systolic and diastolic blood pressure <140/90 mm Hg, respectively, on the same or a reduced number of medications and/or reduced defined daily doses.²

All of the patients were treated with mineralocorticoid receptor antagonists and were normokalemic at the time of adrenalectomy. The procedures followed were in accordance with institutional guidelines;

the protocol was approved by the institutional review committee and adhered to the principles of the Declaration of Helsinki. An informed written consent was obtained from each participant.

Two-Color Microarray-Based Gene Expression

Total RNA was isolated from frozen tissue using RNeasy Mini kit (Qiagen). The integrity and quality of the RNA were systematically checked with a laboratory-on-chip technology in an Agilent Bioanalyzer 2100 with the RNA6000 Nano Assay (Agilent Technologies). The purity was determined by spectrophotometric readings at 260/280/230 nm.¹⁶

Complementary RNA (cRNA) was synthesized from 500 ng of total RNA using the Low RNA Input Linear Amplification kit and the Two-Color RNA Spike-In (Agilent). The Two-Color RNA Spike-In consists of a premixed mixture of 10 transcripts that serve as positive microarray controls to monitor microarray performance (sensitivity, linearity, uniformity, and ratio accuracy). The samples are labeled using cyanine 3 (control pool) and cyanine 5 (APA). Dye swap experiments were included in the experimental design, along with 2 biological replicates to control for gene-specific dye biases and dye intensity differences.¹⁷ The labeled/amplified cRNA was purified using the Qiagen RNeasy mini-spin columns. The purified cRNA was read at the spectrophotometer to get cyanine 3-dye and cyanine 5-cRNA concentrations (picomoles per microliter), absorbance ratios (260/280 nm), and specific activities.

Samples were hybridized onto an oligomicroarray chip (Whole Human Genome Microarray kit, 1×44K, G4112A Agilent) using the Gene Expression Hybridization kit (Agilent) only if the cRNA yield was >750 ng, and the specific activity was >8.0 pmol of cy3 or cy5 per microgram of cRNA. This chip contains ≈44 000 60-mer sequences that include the whole human genome. The chip was incubated in a rotor oven at 65°C for 17 hours and then washed. Slides were scanned using the dual-laser scanner Agilent at 532 and 633 nm to capture fluorescence signals of cy3 and cy5, respectively.

Data Analysis

An expanded Methods section is available in the data supplement available at <http://hyper.ahajournals.org>.

Tumor Classification

We used a Rosetta's classifier algorithm to investigate whether the set of signatures identified by unsupervised analysis could be used to group the tumors. A prognostic algorithm was used by selecting 3 APAs to define each of the tumor classes. A "leave-one-out" cross-validation method, in which 1 sample was left out as a testing sample, and the remaining were used as training data set, was applied to every sample in the data set.¹⁸ Classification accuracy was defined as the percentage of the correct decisions made by the classifier on the testing samples.

Quantitative Real Time RT-PCR

For quantification of the expression of CYP11B2 gene (GenBank ID NM_000498), the TaqMan probe system was used. To avoid

amplification of the 11- β -hydroxylase (CYP11B1), which is highly homologous to CYP11B2 (93% identity by basic local alignment search tool results), the probe was designed in a region specific for the CYP11B2, at position 239 to 262 of the mRNA sequence. For quantification of the gene expression of StAR (GenBank ID NM_000349), SybrGreen chemistry was used. The method used to investigate CAMK-I (GenBank ID NM_003656.3) and CAMK-IIB (GenBank ID NM_172082.1) mRNAs entails a novel real-time RT-PCR that uses Universal ProbeLibrary Probes and Universal ProbeLibrary Assay Design by ProbeFinder Software (Roche). For real-time RT-PCR, we used the Roche LightCycler 480 Instrument. Primers were chosen to span exon-intron boundaries to prevent coamplification of genomic DNA. One microgram of total RNA was reverse transcribed with Iscript (Bio-Rad), and 2 μ L of each reverse-transcription reaction were amplified with specific primers by using LightCycler 480 Probes Master (Roche) in 96 multiwell plates. The mRNA encoding for porphobilinogen deaminase (PBGD; GenBank ID NM_000190) was similarly processed in a separate 1-step RT-PCR for use as housekeeping gene and as a control for RT-PCR performance, as detailed earlier.¹⁹ See the data supplement for sequences of primers and probes, which were selected to allow identical cycle conditions for all of the genes tested: initial denaturation at 95°C for 5 minutes, followed by 55 PCR cycles, each cycle consisting of 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 3 seconds. Quantification of gene expression was carried out by the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$).²⁰

In Situ Hybridization

The tissues were fixed in formalin and included in paraffin. Seven-micrometer-thick sections were mounted on a glass slide. For hybridization of the housekeeping gene (PBGD) transcript, we designed a 92-bp probe labeled with digoxigenin, corresponding with position 406 to 498 of the mRNA sequence. The oligonucleotide probe for the CYP11B2 (39 bp)²¹ was 5' conjugated with digoxigenin. All of the hybridization steps were conducted under RNase-free conditions. For details of the procedure see the data supplement.

Detection of APA Cells in S Phase and in Apoptosis

For details of the procedure see the data supplement.

Statistical Analysis

The statistical analysis was carried out with the SPSS for Windows software (version 14.0, SPSS Italy Inc).

Results

Microarrays Cluster Analysis

By averaging the gene expression values of the 16 APAs, using a 2-fold change from the control pool, 150 sequences were found to be overexpressed and 888 underexpressed ($P \leq 0.05$). We performed an unsupervised analysis, as described in the data supplement, starting from the genes involved in steroidogenesis. Because these tumors feature autonomous excess aldosterone secretion, we expected the expression of genes regulating aldosterone synthesis to be altered in APA cells. By focusing on CYP11B2, we found that its expression profile grouped with CYP11B1, CAMK-I, and 3- β -hydroxysteroid dehydrogenase (HSD3B2; correlation values=0.72, 0.65, and 0.65, respectively; $P < 0.01$ for all).

ANOVA

A 1-way error-weighted ANOVA followed by the Benjamini Hochberg posthoc test was performed to compare gene expression levels across samples and to identify the sequences that differed more across APAs. A total of 16 682

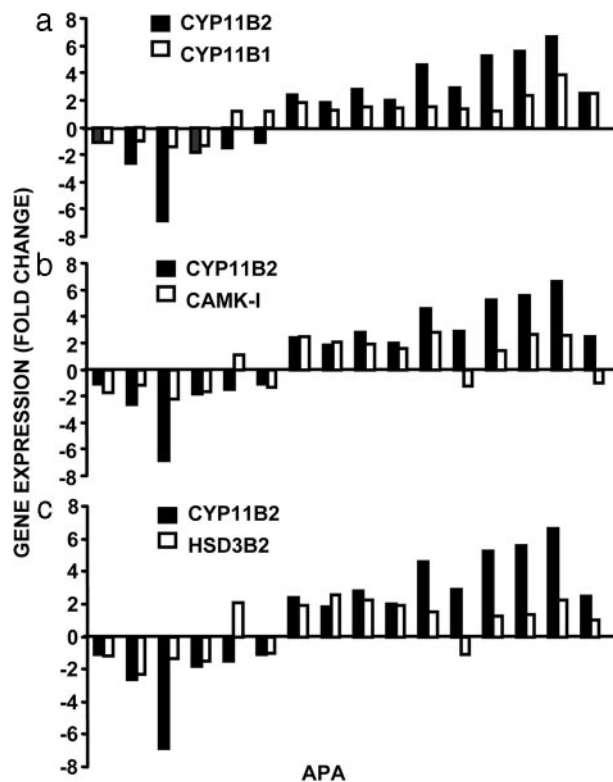


Figure 1. Expression trend analysis of 16 APAs. The expression level of the genes is reported as fold change: the ratio of the intensity of the green channel (APA RNA) to the red channel (control pool RNA). Expression trend of (a) CYP11B2 and CYP11B1 RNA; (b) CYP11B2 and CAMK-I RNA; and (c) CYP11B2 and HSD3B2 RNA.

sequences differed across APAs at $P < 0.05$, indicating tumor heterogeneity. The differentially expressed sequences were composed of the steroidogenic genes CYP11B2 ($P < 0.00001$), CYP11B1 ($P < 0.00001$), HSD3B2 ($P < 0.00001$), 21-hydroxylase (CYP21A2 ($P < 0.00001$), CAMK-I ($P < 0.00001$), and CAMK-IIB ($P = 0.027$).

Trends Analysis

We next evaluated the expression trends of the genes across all of the samples by Trend Analysis, which plots the \log_{10} green signal/red signal of each sequence for each experiment and, therefore, integrates the information given by the cluster analysis, quantifying and plotting the expression values. The signature is defined by a 2-fold increase and a $P < 0.05$. Results will be herein described by groups of genes.

Steroidogenesis Genes

As compared with the normal pool, 17 α -hydroxylase (CYP17A1) was consistently underexpressed in APAs, whereas, at variance, CYP11A1 showed no differences. Unexpectedly, CYP11B2 exhibited a marked heterogeneity of expression across the specimen: 10 APAs showed overexpression, 2 did not differ from the control pool, and 4 showed underexpression of the CYP11B2 gene.

A similar heterogeneity was found in the expression profiles of other steroidogenic enzyme genes, including CYP11B1, HSD3B2, and CYP21A2. Figure 1 shows the trend analysis results for CYP11B2 and CYP11B1 (Figure

1a) and for CYP11B2 and HSD3B2 (Figure 1c). The expression of StAR was significantly lower in APAs than in the control pool.

Calmodulin Cascade Genes

Based on the clustering of CYP11B2 with CAMK-I, we performed a trend analysis of these 2 genes, which confirmed the remarkably similar expression profile of their transcripts (Figure 1b). Among the other members of the calmodulin cascade, a subunit of CAMK-II (CAMK-IIB, transcript variant 6) displayed a trend opposite to that of CYP11B2. The other isoforms, such as CAMK-IV, showed a heterogeneous profile across the samples (data not shown). At variance, there were no differences in calmodulin 1 (CALM1) and 2 (CALM2) gene expression between APAs and the control pool. Of the 2 CAMK kinase members spotted in the array, CAMKK1 and CAMKK2 were not differentially expressed.

Transcription Factors Involved in the Regulation of Aldosterone Synthesis

The nuclear receptor NURR1 (NR4A2) showed a heterogeneous profile across the samples, without any relationship with the CYP11B2 expression. Among the CREB isoforms, CREB1 and CREB5 were also found to be heterogeneously expressed; CREB3 was not consistently different from the control pool. The steroidogenic factor-1 (SF-1; NR5A1), a known repressor of CYP11B2 expression, and NGFIB were not differentially expressed as compared with the pool. Expression of ATF-1, an activator of the aldosterone synthase transcription, was not different in APAs as compared to the control pool. DAX1 was overexpressed in 13 of 16 APAs; however, it showed no relation with the CYP11B2 expression.

Biological Replicates Analysis

Biological replicates of 4 samples were also analyzed. We extracted RNA from different portions of 2 tumors featuring lower CYP11B2 expression, from one with a higher expression and from one with no difference, as compared with control pool. The profile of CYP11B2 was remarkably similar in the first 3 cases, but differed in the last, where CYP11B2 resulted to be overexpressed.

Tumors Classification and Cross-Validation

We used a classifier algorithm to investigate whether the set of genes identified by trend analysis as potentially useful discriminators between groups could indeed provide an accurate grouping of the tumors. We selected 200 sequences with an expression trend similar to that of CYP11B2 to build the sequence set to be used for tumor classification. Among the others, these sequences were composed of CYP11B1, HSD3B2, CAMK-I, and CYP21A2.

The 2 tumor classes were defined using the expression profile of the groups of 3 APAs with the extreme differences in CYP11B2 expression. All of the remaining 10 APAs, used as the “training data set,” were correctly classified: 3 were in the group featuring low steroidogenic genes expression, and 7 were in the other group (Figure 2). The leave-one-out cross-validation confirmed these results and also provided a

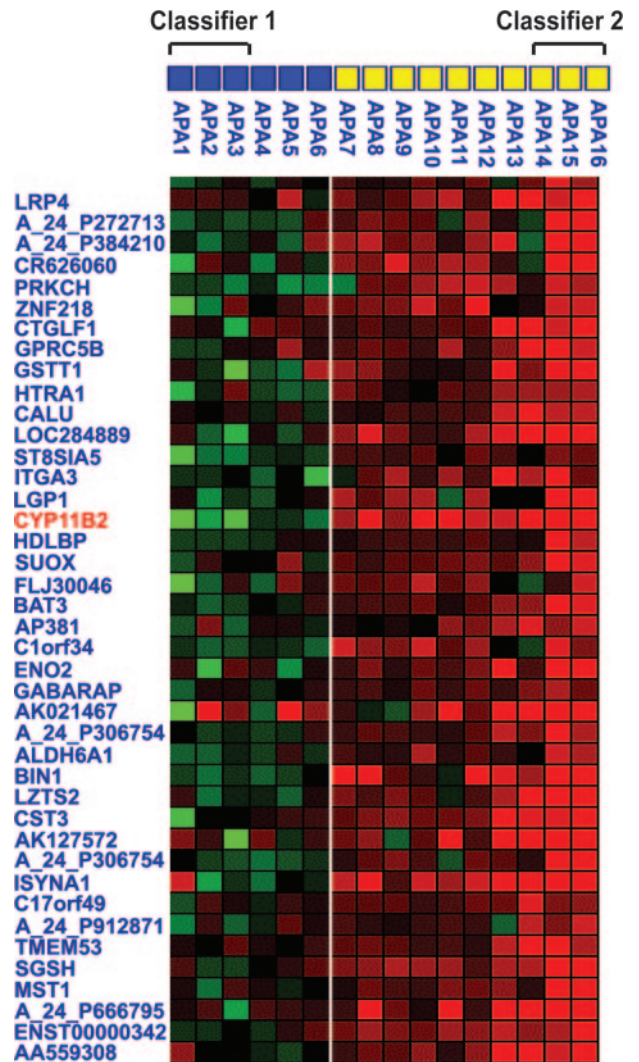


Figure 2. Example of tumor classification. A prognostic algorithm was applied using 3 APAs with the most extreme differences of gene expression profile (identified as classifier 1 and 2) to allocate the remaining APAs to the 2 tumor classes. Two-hundred sequences with an expression trend similar to that of CYP11B2, composed of, among others, CYP11B1, HSD3B2, CYP21A2, and CAMK-I, allowed to correctly classify the remaining 10 APAs.

high classification accuracy (of ≈94%), with only 1 APA misclassified.

Clinical Features

To investigate whether a different molecular signature was associated with clinically relevant phenotypic differences, we compared the 10 APAs with a high to the 6 with a low CYP11B2 expression for age, gender, severity and duration of hypertension, plasma renin activity, plasma aldosterone concentration, and tumor size (Table). We found no significant differences of all of these variables with few exceptions: the low CYP11B2 had a longer known duration of hypertension (142.3±45.6 months versus 90.0±45.6 months; P=0.016). Moreover, although all of the patients were, by definition, cured or markedly improved by adrenalectomy, the low CYP11B2 resulted to be less likely to be cured than the high CYP11B2 patients (χ²=6.15; P=0.013).

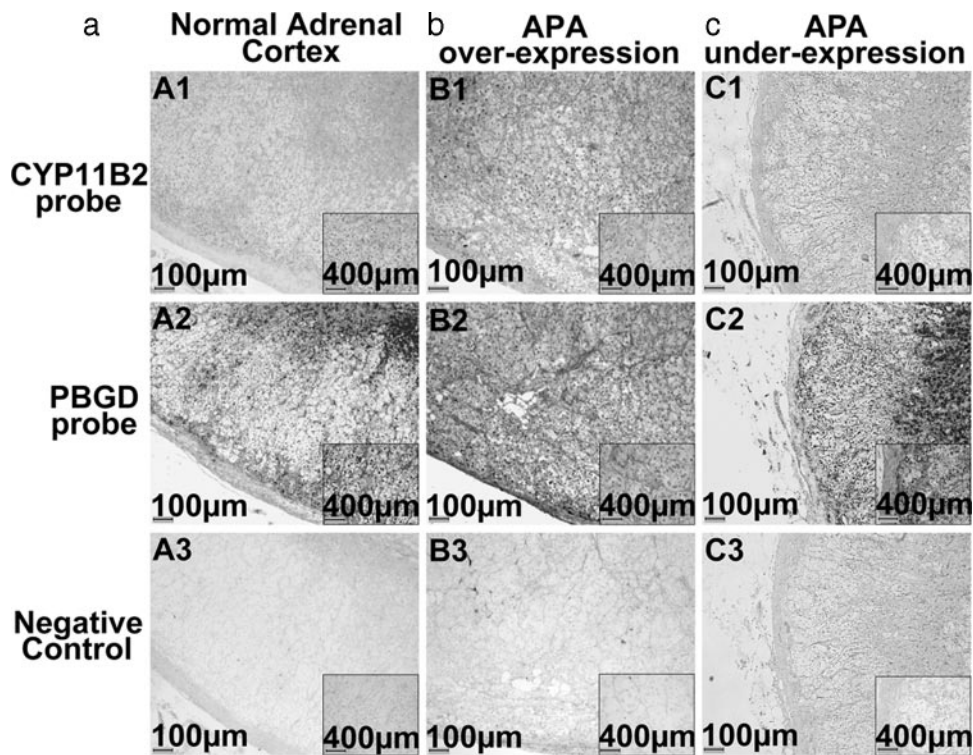


Figure 3. Exemplary results of an in situ hybridization experiment for CYP11B2 and the housekeeping gene PBGD. a, Normal human adrenal cortex; b) APA showing overexpression of CYP11B2, CYP11B1, HSD3B2, CYP21A2, and CAMK-I; c) APA exhibiting underexpression of CYP11B2, CYP11B1, HSD3B2, CYP21A2, and CAMK-I. In the bottom panels, the molecular probes for CYP11B2 or PBGD were omitted, but the sections were identically processed, thus providing negative controls for the hybridization reaction.

Quantitative Real-Time RT-PCR Results

We used quantitative real-time RT-PCR to validate the oligomicroarray data.²² We quantified the transcript levels of 4 genes with the $\Delta\Delta C_t$ method, where the gene expression is reported as the percentage of change from the control pool. Calibration curves were built for each gene analyzed, starting from 1 µg of control RNA and serially diluting the samples 1:5 five times. Each dilution was run in 2 replicates. The PCR results fully confirmed the heterogeneity of the expression profiles of CYP11B2, CAMK-I, and CAMK-IIB (data not shown), as well as the opposite trend of CAMK-IIB versus that of CYP11B2 and CAMK-I in many APAs. The underexpression of StAR was also confirmed (0.84 ± 0.02 ; data not shown).

In Situ Hybridization Results

In situ hybridization (Figure 3) for the housekeeping gene PBGD showed a clear-cut staining throughout the normal human adrenal cortex (Figure 3a) and the APAs (Figure 3b and 3c). Use of the CYP11B2-specific probe highlighted marked differences of staining between the APAs featuring a high (Figure 3b) and a low CYP11B2 gene expression (Figure 3c) at oligomicroarray analysis. The staining for both CYP11B2 and the housekeeping gene was predominantly nuclear, a finding consistent with a preservation of the transcript of interest at this level. No staining was observed when the molecular probes for either PBGD or CYP11B2 were omitted (Figure 3, bottom panels).

Phase S and Apoptosis Cell Detection in APA

By this approach, we investigated whether the differences of gene expression profile across APAs might be related to a different proportion of APA cells undergoing proliferation and apoptosis in different regions of the tumor. Results showed that cells undergoing S phase and apoptosis coexisted within the same tumor (Figure 4) and that the APA cells showing CYP11B2 gene expression could be found more easily where apoptotic cells were also located. Thus, in APAs, a proliferating phenotype was associated with a low or absent CYP11B2 gene expression, and a steroid-secreting phenotype was related to an apoptotic cell phenotype.

Discussion

We investigated the molecular mechanisms leading to aldosterone excess in APA with a novel high-throughput technique, the high-density oligonucleotide microarrays. By comparing the whole transcriptome of APAs with a pool of histologically normal subcapsular adrenocortical tissues, we sought for differentially expressed genes and molecular differences and similarities of the tumor signatures that can help to classify them into homogenous groups. The transcriptome profile was also compared across APAs with validated bioinformatics tools, as a cluster and trends analysis. Real-time quantitative RT-PCR assays confirmed the microarray results for the genes of interest, indicating their robustness. With this powerful molecular approach, we could show that, although the APAs present with the same phenotypic features, eg, arterial hypertension and autonomous aldosterone

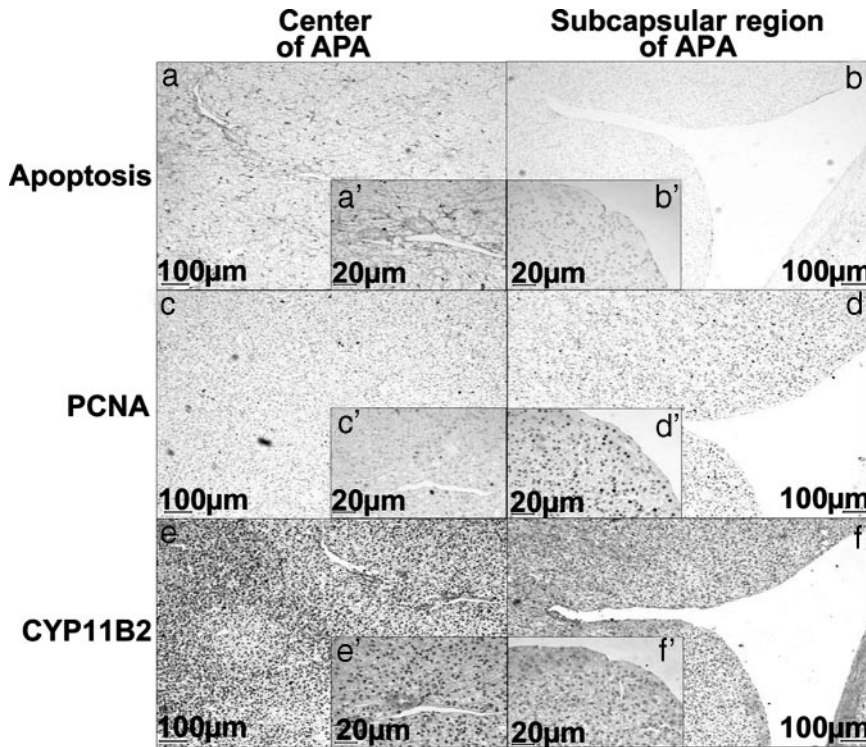


Figure 4. Exemplary results of the experiments for assessing apoptosis and S-phase cell in APA sections and for in situ hybridization of CYP11B2. Different portions of an APA were examined: the center (a, c, and e) and the subcapsular regions (b, d, and f). Apoptotic cells (a and b), S-phase cells (c and d), and cells expressing aldosterone synthase by in situ hybridization of CYP11B2 (e and f) were identified in adjacent sections of APA. See text for explanation.

production, the expression profile of steroidogenic genes markedly differs across tumors.

Using information on differentially expressed genes, we could identify 2 subgroups of APAs characterized by divergent trends of expression of genes, as CYP11B2, CYP11B1, HSD3B2, and CYP21A2, that are involved in aldosterone and cortisol synthesis. Hence, these findings allow us to outline 2 hypothetical molecular scenarios in APAs: the first features an overexpression of steroidogenic enzymes (shown in black in Figure 5a), leading to overproduction of aldosterone, and the second shows a paradoxical underexpression of CYP11B2 and other steroidogenic enzyme genes (shown in white boxes in Figure 5b), as compared with normal adrenocortical tissue.

These results also suggest that there is a poor correlation between the overall aldosterone secretion, which can be estimated by plasma aldosterone levels, and the CYP11B2

transcript levels measurable in a tumor tissue specimen. The lack of significant differences in plasma aldosterone levels between the high and low CYP11B2-expressing APA groups supports this contention.

Interestingly, the high CYP11B2 patients had a significantly shorter known duration of hypertension and were more likely to be long-term cured than the low CYP11B2 patients, suggesting that the molecular signature of APA might carry some prognostic information and/or that the long-standing hyperaldosteronism, with ensuing vascular remodeling and organ damage, can affect the gene expression profiles of APA. This issue is worthy of further investigative efforts in a larger series of tumors.

The CYP11B2 underexpression might seem to conflict with the high plasma aldosterone levels that characterize APA patients and the reported high CYP11B2 gene expression.^{4,5,14} However, as the APA is composed of a much larger

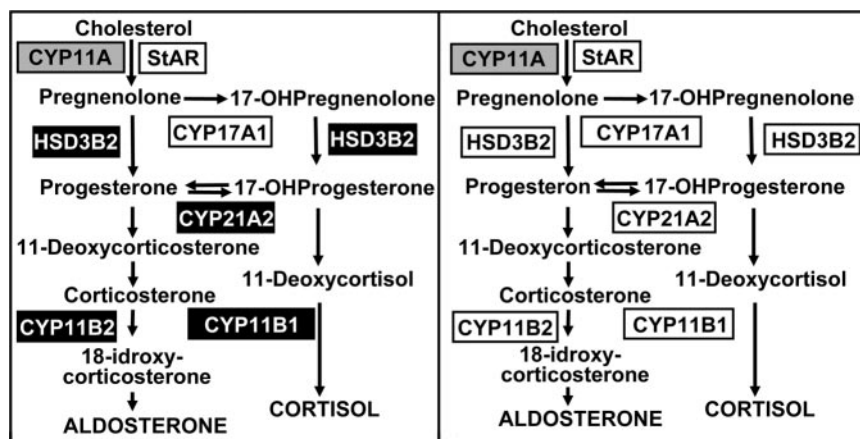


Figure 5. Schematic representation of the differential expression of steroidogenic enzyme genes in APAs showed by microarray technique. In black are genes overexpressed, in white genes underexpressed, and in gray genes not differentially expressed. Two APAs subgroups were outlined: the first features an overexpression of steroidogenic enzymes RNA (a), the second a low expression (b). CYP11A indicates cytochrome P450 side chain cleavage enzyme.

number of aldosterone-secreting cells than the normal zona glomerulosa, a high secretion of aldosterone and a normal, or even low, CYP11B2 expression should not be seen as contrasting conditions: even if the production of aldosterone per single APA cell is small, the overall aldosterone synthesis can still be several folds higher in an APA than in a normal adrenal gland. Previous studies might have failed to pinpoint the heterogeneity of CYP11B2 expression in APAs for several reasons, including a selection bias, because of the specimen of tumor being examined (see below), the small number of APA examined, the selection of only APAs with a high CYP11B2 expression, and the pooling of tumor tissues.^{5,14}

Overall, our data suggest that the mechanisms underlying the aldosterone excess in APAs are far more complex than originally thought. In accord with previous data,¹⁴ we found no alterations in the expression of the CYP11A1 gene encoding the first enzyme of the steroidogenic cascade. This suggests that this gene does not entail a molecular signature of aldosterone overproduction in APAs. The underexpression of the gene encoding CYP17A1, the first enzyme involved in cortisol biosynthesis, along with the high level of CYP11B1 found in some APAs, is consistent with the view that, in the APA cells, aldosterone synthesis prevails over cortisol production; even when CYP11B1 is highly expressed, cortisol biosynthesis is upstream limited; and the CYP11B1 can supply corticosterone as a substrate for aldosterone biosynthesis.

Our results also suggest that the excess aldosterone production does not involve enhanced expression of StAR, because this gene was found to be consistently underexpressed in APAs. However, the possibility that the activity of StAR can be upregulated by posttransductional mechanisms, as phosphorylation of regulatory domains, remains to be excluded in further specific research.

This study shows for the first time in APAs *ex vivo* that the expression profile of CAMK-I, a regulator of CYP11B2 transcription, mirrors that of CYP11B2 (Figure 1b). This is of interest because CAMK-I enhances CYP11B2 expression⁸ through the phosphorylation of the CREB transcription factors family. Thus, a high CAMK-I expression may explain the increased CYP11B2 gene transcription found in $\approx 60\%$ of the APAs. The lack of a relationship between CYP11B2 levels and others known regulators of its transcription, such as NURR1 and the CREB family members, SF-1 and NGFIB, suggests that the transcriptional activity of these factors is predominantly modulated by posttransductional, rather than by transcriptional, mechanisms.

CAMK-II is composed of 4 different chains, α , β , γ , and δ , which assemble into homomultimeric or heteromultimeric holoenzymes made of 8 to 12 subunits to give the different isoforms. CAMK-IIB codes for the β -chain of the kinase, which is widely expressed in tissues (GeneCard database, Weizmann Institute of Science, www.genecard.org). CAMK-II plays a direct role in the agonist-stimulated production of aldosterone by enhancing cholesterol delivery to the mitochondria outer or inner membrane.¹⁰ Interestingly, CAMK-IIB was overexpressed in 75% of our APAs with underexpression of CYP11B2 and CAMK-I and underexpressed in those with overexpression of CYP11B2 and

CAMK-I. Thus, it is likely that the excess aldosterone synthesis in the first APA subgroup occurs via an increased CAMK-II-mediated signaling. Consistent with this contention, it has been hypothesized that CAMK-II is implicated in phosphorylation of StAR residues, which may be crucial for the activation of this transporter, or in the facilitation of cholesterol transfer via the regulation of cytoskeleton proteins.¹⁰ A higher amount of aldosterone precursors in the mitochondria fueling aldosterone biosynthesis rate, even despite no overt increase of CYP11B2 transcripts, can be the ultimate result of these processes.

Finally, our results provide evidence for a new molecular classification of APA, based on a widely accepted gene class discovery approach.^{23,24} We classified APAs using a classifier algorithm (Rosetta Classifier) with 100% of classification accuracy. Our class definition was cross-validated using a leave-one-out approach with a 94% of accuracy.

Potential Limitations of the Study

Because of the impossibility of obtaining a homogeneous population of normal human zona glomerulosa cells, we used normal subcapsular adrenocortical tissues as a referent. This tissue is composed of not only zona glomerulosa, but also of other cell phenotypes, including vascular cells. Thus, comparison of the relatively homogeneous APA tissue with this heterogeneous subcapsular tissue would be expected to cause, if any, an overassessment of the expression of genes involved in aldosterone production in APAs. Our finding of no consistent CYP11B2 overexpression in APAs, therefore, supports the conclusion that enhanced CYP11B2 transcription is not a molecular signature of APA and that its expression is heterogeneous across these tumors. Results obtained with biological replicates and *in situ* hybridization also suggest that aldosterone synthesis is heterogeneous across APAs and even within the same APA, with cells featuring different degrees of aldosterone synthase gene expression. By using mRNAs extracted from different histologically identified portions of 2 low, 1 high, and 1 not differentially expressing CYP11B2 APAs, we could confirm the CYP11B2 expression profile in the first 3 but not in the latter case. *In situ* hybridization (Figure 3) confirmed the existence of APAs featuring a high and low CYP11B2 expression and evidenced some degree of heterogeneity, even within the same tumor. To gather insight on within-tumor heterogeneity, we investigated the proportion of APA cells undergoing proliferation and apoptosis in different regions of the tumor: not only did we find that cells undergoing S phase or apoptosis coexisted within the same tumor (Figure 4) but also that the APA cells with a higher CYP11B2 gene expression could be found more easily in areas with apoptotic cells. These results suggest that a proliferating phenotype is associated with a low or absent CYP11B2 gene expression, whereas steroid-secreting phenotypes localize in areas enriched with an apoptotic cell phenotype. Hence, overall, the results of transcriptome studies in APAs can depend, to some extent, on the tissue specimen being examined, an issue that needs to be considered in future works in this area. Finally, it is worth mentioning that transcript levels can be a poor surrogate for the functional status of enzymes and, therefore,

that further studies aimed at measuring enzyme protein levels and activity are needed to conclusively support these findings.

Perspectives

APAs feature a heterogeneous expression of the steroidogenic enzymes when assessed with a whole transcriptome approach. This heterogeneity allows a classification of tumors into molecularly homogeneous groups of patients, who exhibit differences in the duration of hypertension and also in the likelihood of being long-term cured with adrenalectomy. Thus, whereas complex molecular mechanisms underlie the excess aldosterone production in these endocrine tumors, future research should be aimed at verifying whether the differences identified at the mRNA levels are paralleled by changes at the protein and enzyme activity levels. Moreover, the molecular mechanisms of the APA heterogeneity as a function of the in vivo and in vitro responses to functional stimuli and of the long-term response to adrenalectomy need to be further dissected.

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Disclosures

None.

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Heterogeneity of Aldosterone-Producing Adenomas Revealed By A Whole Transcriptome Analysis

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Supplemental Methods

Microarray Data Analysis. After checking the spots in a TIFF file for imperfections scan data were loaded into Feature Extraction version 9.1 (Agilent) for analysis. Algorithms were then applied to 1) define features and background regions, 2) flag features, background regions and pixels that may affect the reliability of the results, 3) subtract background information from the features, and 4) normalize the signals for any differences in dye signal intensity. Outliers were flagged and then removed from the analysis. To decrease systematic biases background signal was subtracted from the raw signal. Moreover, to correct differences in dye bias, we applied the “linear & lowess method” which performs a linear normalization across the entire range of data and then applies a non-linear normalization to the linearized data set.^(1;2)

After extraction, results were loaded into Rosetta Resolver (Rosetta Biosoftware, Seattle, WA) to compare data from different microarray experiments. An unsupervised analysis was performed, which is a data-mining technique that does not need any previous information and search for transcripts with similar behavior by using grouping techniques.⁽³⁾ We first identified the up-and down-regulated genes in each array as compared to the normal subcapsular adrenocortical control tissue. Then a comparison across APAs was performed to reveal the differentially expressed genes in APAs. To determine similarities (or differences) across arrays and/or sequences we used the ‘Clustering’ function and ‘Trending data’.

In situ Hybridization. Sections of 8 APAs were dewaxed with xylene (5min for 2 times), and re-hydrated in different ethanol concentrations (5 min for 2 times), quickly washed in DEPC-dH₂O and phosphate buffered saline (PBS, Oxoid) (5 min for 2 times). After post-fixing the sections for 10 min with 4% PFA in PBS and washing in PBS (5 min for 2 times), they were acetylated for 5 min with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine (TEA, Sigma) pH 8.0 and subsequently with acetic anhydride (Fluka, Italy) 0.25% (v/v) in TEA and in SSC 2X (Sigma, Italy) (3 min). The sections were treated with proteinase K (Sigma), 10 µg/ml in PBS, at 37°C for 20 min

and then immersed in glycine (Fluka), 2 mg/ml in PBS, at 4°C for 1 min 30 seconds and PBS (5 min for 2 times). The sections were incubated for 2 h at 37°C with pre-hybridization buffer and washed in SSC 2X (5 min), then incubated with hybridization solution containing DIG-labeled probes in hybridization buffer (50% deionized formamide (Sigma), 20% (w/v) dextran sulfate, 1% Denhardt's 50X solution (Sigma), 20% SSC 2X, 1% Dithiothreitol (DTT, Fluka) and 1% Salmon sperm DNA (19mg/ml, Invitrogen, Italy) at 37°C overnight. Slides were washed with DTT (1.5mg/ml)/SSC 1X at room temperature (5 min), 55°C (2 times for 15 min) and DTT (1.5mg/ml)/SSC 0.5X at 55°C (10 times for 2min), at room temperature (5min) and then transferred to Tris-buffered saline (TBS) (100 mM Tris HCl, 150 mM NaCl, pH 7,5) and washed 5 min for 3 times. The sections were covered with blocking solution (TBS, 0.1% tritonX-100 and 1% sheep serum, Sigma) at room temperature for 30 min and subsequently incubated with anti-DIG antibody (Roche, Italy) diluted 1:2000 overnight at 4°C. After washing the sections 3 times for 5 min in TBS, they were incubated with staining solution (Fast™ NBT/BCIP in 10 ml of dH₂O add 1% levamisol 1M, Sigma) overnight at room temperature.

Detection of APA cells in S phase and in apoptosis. 7 µm-thick sections of tissues fixed in formalin and included in paraffin were mounted on glass slide. For the detection of S phase cells sections were de-waxed three times in xylol, re-hydrated in a graded series of ethanol and unmasked with Antigen unmasking buffer (DIAPATH, Bergamo, Italy) at 96 °C for 15 min. Endogenous peroxide was blocked with hydrogen peroxide (3%) in methanol for 5 min; non-specific background staining was minimized with blocking serum solution (BSA 0,2% triton 0,2% normal goat serum 1:50 in PBS) for 20 min. Sections were then exposed to Proliferating Cell Nuclear Antigen (PCNA) antibody (Santa Cruz Biotechnology – Cat. Number: SC-53407) diluted 1:100 in BSS and incubated overnight at 4°C. After incubation, secondary antibody (IgG or IgM Anti Mouse/Rabbit polyclonal HRP, Chemicon International, U.S.A) was added for 1 h at room

temperature and AEC (Immuno Pure AEC PIERCE Illinois, U.S.A) 0,4% in Sodium Acetate-Acetic Acid Buffer 0,1M pH 5,2 was used for visualization of peroxidase. Negative controls were identically processed, but with omission of the primary antibody. To detect apoptotic cells in APAs we used a method (TdT-FragEL™, Calbiochem, Germany) that detects DNA fragmentation. After de-waxing and re-hydration as described above, sections were permeabilized with proteinase K (Sigma), 10 µg/ml in Tris pH 8 for 15 min at 37°C, and exposed to hydrogen peroxide (3%) in methanol for 5 min to block endogenous peroxide. The sections were equilibrated with 1X TdT at room temperature for 30 min and treated with 57 µl of TdT Labelling Reaction Mix and 3 µl of TdT Enzyme at 37°C for 120 min. The reaction was stopped with the Stop Solution (EDTA 0,5 M) at room temperature for 5 min. Sections were then exposed to 50X Conjugate (Peroxidase-Streptavidin) diluted 1:50 with Blocking Buffer (4% bovine serum albumin in PBS) for 30 min at room temperature. For visualization we used 3,3-diaminobenzidine Tablets (Sigma) in water. Negative controls were obtained by replacing the TdT with dH₂O during the labelling step. Sections were counterstained with methyl green. Thus, apoptotic and non apoptotic cells were identified by a brown and a green staining of the nuclei, respectively.

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Supplemental Table

Table S1. Real Time RT-PCR primer sequences and amplicons

Primers	Sequence	Accession number	Amplicon
CYP11B2		NM_000498	108 bp
Sense	5'-AGATGCACCAGACCTTC-3'		
Antisense	5'-CTGTTGCAGCTTCTCCACATC-3'		
Probe	5' -TCAGGTACAACCTGGGAGGACCAC-3'		
CAMK-I		NM_003656.3	61 bp
Sense	5'-AAGGCAGCATGGAGAATGAG-3'		
Antisense	5'-GCTACAATGTTGGGGTGCTT-3'		
UPL	5'-TGCTGTCC-3'		
CAMK-IIB		NM_172082.1	112 bp
Sense	5'-CTCAGTGGGCAGACAGACC-3'		
Antisense	5'-TCCATCTGCTTTCTTGTTGAGTA-3'		
UPL	5'-TGGTGGAA-3'		
PBGD		NM_000190	92 bp
Sense	5'- TGCCCTGGAGAAGAATGAAG-3		
Antisense	5'- AGATGGCTCCGATGGTGA-3'		
UPL	5'- CTTCCTCC-3'		
StAR		NM_000349	647 bp
Sense	5'-TGTCATCAGGGCGGAGCA -3'		
Antisense	5'- CTCTGAGATTCTGCTTTGTGC-3'		