**BEX1** is differentially expressed in aldosterone-producing adenomas and protects human adrenocortical cells from ferroptosis

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Abstract

Aldosterone-producing adenomas (APA) are a major cause of primary aldosteronism. Somatic mutations in ion channels and transporters drive the aldosterone overproduction in the majority of APAs with mutations in the KCNJ5 G protein-coupled potassium channel predominating in most reported populations. Our objective was to gain insight into biological mechanisms of APA tumorigenesis by comparing transcriptomes of APAs of distinct sizes by mRNA sequencing analysis (9 APAs with adenoma diameter ≥30 mm versus 12 APAs ≤10 mm). Genes with significantly altered expression levels between these 2 groups were identified in APAs with no mutation detected (NMD, 348 genes) and with a KCNJ5 mutation (155 genes). We validated the differential expression of 10 genes with a known function related to cell death and proliferation in an expanded sample set of 71 APAs by real-time qPCR (58 macro-APAs, diameter≥10 mm; 13 micro-APAs, diameter<10 mm). We focused on BEX1 that was upregulated in micro-APAs relative to macro-APAs (2.76-fold, P<0.001) and compared with paired adrenal cortex (3.85-fold, P<0.05), and showed a linear negative correlation with APA diameter in the NMD group (r=-0.501, P=0.007). Compared with control cells, stable expression of BEX1 in human adrenocortical cells did not alter cell cycle progression or sensitivity to apoptosis but conferred protection from ferroptosis (P<0.01), a form of regulated cell death, measured by flow cytometry. Taken together, these findings demonstrate that BEX1 promotes cell survival in adrenal cells by mediating the inhibition of ferroptosis and suggest a function for BEX1 in the pathogenesis of APAs.

Key words: aldosterone, cell death, primary aldosteronism, proliferation, regulated necrosis, tumorigenesis
Introduction

Primary aldosteronism (PA) is the most frequent surgically correctable cause of hypertension. The unilateral forms of the disease are mainly caused by an aldosterone-producing adenoma (APA) and are specifically treated and potentially cured by adrenalectomy.\(^1\)\(^2\) The surgically removed adrenals have been used to demonstrate the presence of somatic mutations in APAs in genes encoding ion channels (\(\text{KCNJ5}\), \(\text{CACNA1D}\), \(\text{CACNA1H}\), \(\text{CLCN2}\)) and transporters (\(\text{ATP1A1}\), \(\text{ATP2B3}\)). These mutations disturb ion homeostasis and activate \(\text{Ca}^{2+}\) signaling resulting in increased expression of \(\text{CYP11B2}\) (encoding aldosterone synthase) and constitutive aldosterone production.\(^6\)\(^9\) The use of targeted next-generation sequence analysis guided by \(\text{CYP11B2}\)-immunohistochemistry of paraffin-embedded adrenals has greatly increased the detection of somatic mutations in APAs to achieve a combined prevalence of over 90%.\(^10\)\(^12\)

The role of the above mutations in cell death and proliferation is less clear. Activating somatic mutations in \(\text{CTNNB1}\), encoding \(\beta\)-catenin, have been identified in APAs.\(^13\) The prevalence of \(\text{CTNNB1}\) mutations in APAs is lower than in other adrenocortical tumors\(^14\) but the proportion of APAs with constitutive Wnt/\(\beta\)-catenin signaling is high\(^15\) implicating other factors in the activation of this pathway. Different \(\text{KCNJ5}\) mutations have diverse effects on adrenocortical cell growth\(^16\) depending on the level of \(\text{Na}^{+}\) conductance that determines the degree of cell toxicity.\(^3\)\(^17\)

We hypothesized that transcriptome profiling of APAs of distinctly different diameter may highlight genes that function in cell death and proliferation. We used mRNA sequencing (mRNA-seq) to compare the transcriptomes of small and large APAs from patients with PA.
with a similar known duration of hypertension. Our objective was to identify novel genes and biological mechanisms involved in the deregulated cell growth of adrenal cells and translate these findings to a potential role in APA pathogenesis.

Materials and Methods

The authors declare that all supporting data are available within the article and its online supplementary files. mRNA-seq data have been made publicly available and can be accessed at https://github.com/MedIVLMUMunich/MacroMicroAPA_RNAnseq

Patient samples

The study comprised 71 APAs surgically removed from patients diagnosed with unilateral PA at 2 referral centers (39 from the Medizinische Klinik IV, Klinikum der Ludwig-Maximilians-Universität München, Munich, Germany and 32 from the Hypertension unit, Department of Medical Sciences, University of Torino, Turin, Italy). For 17 APAs, the corresponding adjacent cortex was also available. PA was diagnosed according to current guidelines1-18 including adrenal venous sampling for subtype differentiation of unilateral from bilateral PA.19 All patients included in the study displayed complete biochemical success after surgery according to the PASO criteria confirming the diagnosis of unilateral PA.2,20 The study also included 20 cortisol-producing adenomas and 8 incidentalomas diagnosed at the Munich center. Adenoma diameters were determined from the largest nodule at pathology. Research protocols were approved by local ethics committees, and all participants provided written informed consent.
**Sanger sequencing of genomic DNA**

*KCNJ5* and hot spot regions of *ATP1A1, ATP2B3, CACNA1D* were sequenced by Sanger sequencing of PCR-amplified genomic DNA extracted from fresh frozen nodules resected from patients with APA as described previously.\(^{21}\) *CTNNB1* was sequenced using primers shown in Table S1. The histopathology of all formalin-fixed paraffin embedded adrenals was evaluated using a specific CYP11B2 monoclonal antibody\(^{22}\) to confirm the presence of an APA or an aldosterone-producing nodule in the resected gland.\(^{23}\)

**Next generation sequencing and bioinformatics analysis**

mRNA-seq transcriptome profiling was performed of 21 APAs comprising 9 large macro-APAs with adenoma diameter ≥30 mm (5 with a *KCNJ5* mutation and 4 with NMD) and 12 micro-APAs with adenoma diameter ≤10 mm (6 with a *KCNJ5* mutation and 6 with NMD). mRNA-seq was performed by QIAGEN Genomics (Hilden, Germany). Heatmap and unsupervised hierarchical clustering and volcano plots were performed using R or the ClustVis web tool (https://biit.cs.ut.ee/clustvis/).\(^{24}\)

**Cell lines and culture conditions**

Human adrenocortical (HAC15) cells\(^{25}\) (a kind gift from Professor William E. Rainey, University of Michigan, Ann Arbor) were used to establish stable cell lines as described previously.\(^{16}\)

**Flow cytometry**

Vibrant DyeCycle Violet stain was used for cell cycle analysis. Propidium iodide (PI) was used to quantify proportions of PI-positive dead cells following ferroptosis induction (with 4 µM \(1S,3R\)-RSL3 [RSL3]) in the presence or absence of ferroptosis inhibitor (10 µM liproxstatin-1,
Cell populations were detected on a FACSCalibur (BD Biosciences) or a BD Accuri C6 flow cytometer with FL2 detector (BD Biosciences). Data were analyzed with FLowJo version 10.4. All experiments were performed in triplicate, with a minimum of 7000 (cell cycle experiments) or 15000 (ferroptosis experiments) single cells analyzed per sample.

**Statistical analysis**

Statistical analyses were performed using IBM SPSS version 25.0 and GraphPad Prism version 8.2.1. Data were analyzed using the Kolmogorov-Smirnov test and Shapiro-Wilk test to determine distributions. Statistical significance was assessed by a t-test (paired where appropriate) or a Mann-Whitney test (Wilcoxon rank matched pairs test if needed) or a Bonferroni’s post-test after two-way ANOVA. Chi-square and Fisher’s exact tests were used to compare categorical variables. Univariate correlations were assessed using Pearson correlations. P values <0.05 were considered statistically significant.

**Results**

**mRNA-Seq Transcriptome Analysis of APAs**

Tumor samples used for mRNA-seq analysis (21 APAs) displayed a median nodule diameter of 34.0 mm [32.5-37.5] and 7.5 mm [6.3-10.0] (P<0.001), in each group (APAs ≥30 mm versus APAs ≤10 mm diameter). There were no significant differences between the 2 groups in known duration of hypertension (Table S2).

Unsupervised hierarchical clustering of 500 genes with the largest coefficient of variation classified four gene clusters categorized by genotype and adenoma diameter (Figure 1A). Differential expression analysis identified 348 and 155 significantly altered genes in the NMD
and KCNJ5 subgroups, respectively (Figure 1B, 1C). Specifically, there were 119 upregulated and 229 downregulated DEGs in APAs with NMD, and 54 upregulated and 101 downregulated DEGs in the KCNJ5 subgroup. The top 20 upregulated and downregulated genes in APAs (diameter ≥30 mm versus ≤10 mm) with NMD and with KCNJ5 mutations are shown in Tables S3 and Table S4, respectively. The complete list of DEGs can be downloaded for: the total dataset:

https://github.com/MedIVLMUMunich/MacroMicroAPA_RNAseq/raw/main/Total_DEGs.xlsx

; and stratified by APAs with NMD:

https://github.com/MedIVLMUMunich/MacroMicroAPA_RNAseq/raw/main/NMD_DEGs.xlsx

; and with a KCNJ5 mutation:

https://github.com/MedIVLMUMunich/MacroMicroAPA_RNAseq/raw/main/KCNJ5_DEGs.xlsx

**Gene Ontology Enrichment Analysis**

DEGs were annotated to Gene Ontology (GO) terms of biological processes that identified different patterns of gene set enrichment between macro- and micro-APAs according to genotype (APAs with NMD and with a KCNJ5 mutation). Cell death was the most significantly enriched biological process in APAs with NMD \((P=6.4\times10^{-8})\) (Figure S1A). Other enriched GO terms in this group were related to axon guidance, angiogenesis, cell migration, the cellular response to zinc ions or Wnt signaling (Figure S1A). APAs with KCNJ5 mutations showed enrichment of genes related to cholesterol biosynthesis, the cell cycle and cell division. Other enriched terms in this group were associated with RNA processing, signal transduction and organization of cellular components (Figure S1B).
**DEGs Involved in Cell Death and Proliferation**

Unsupervised hierarchical clustering of the top 40 DEGs associated with cell death and proliferation identified by the mRNA-seq analysis categorized gene clusters according to adenoma diameter in the NMD and KCNJ5 subgroups (Figure S2). In the mRNA-seq analysis, no ferroptosis suppressor was differentially expressed in APAs with KCNJ5 mutations. Conversely, 2 DEGs MT1G (metallothionein 1G) and CAV1 (caveolin 1), encoding ferroptosis inhibitor proteins, were identified in the NMD subgroup (log2[APA ≥ 30 mm/ ≤ 10 mm] = 3.10, adjusted P value = 0.0068; log2[APA ≥ 30 mm/ ≤ 10 mm] = -1.87, adjusted P value = 0.0415) (Figure S3).

The expression levels of a subset of DEGs (identified by mRNA-seq analysis) with a reported role in cell death and proliferation, were determined in an expanded sample set of APAs (n=71; median diameter 15.0 mm [11.0-21.0]) (Table 1). APAs in this sample set were stratified into macro- and micro-APAs according to a cut-off diameter of 10 mm (macro-APAs [diameter ≥ 10 mm], n=58, median diameter, 16.0 mm [14.0-22.8]; micro-APAs [diameter <10 mm], n=13, median diameter, 7.0 mm [6.5-8.0], P<0.001). The macro-APA group comprised a higher proportion of women (63.8% and 30.8% in the macro- and micro-APA groups, respectively, P=0.029) and APAs with a KCNJ5 mutation (67.2% in macro-APAs versus 30.8% in micro-APAs, P=0.015). The micro-APAs included a higher proportion of APAs with NMD (69.2% versus 32.8%) (Table 1).

Figure 1D shows the relative expression levels in macro- versus micro-APAs of 10 genes with a known role in cell death and proliferation. These genes included those with a role in β-catenin signaling (mRNA levels in macro- versus micro-APAs: TSPAN12, -8.30-fold, P<0.0001;
SFRP2, -5.85-fold, P<0.001; DKK1, -1.77-fold, P<0.01). TSPAN12, BEX1, FBXL21, and TMPRSS3 gene expression levels were weakly correlated with adenoma diameter in the combined group of APAs (APAs with NMD+APAs with KCNJ5 mutations), stronger correlations were observed in APAs with NMD (Table 2). In the NMD group, FBXL21 and TMPRSS3 gene expression was strongly positively correlated with adenoma diameter (FBXL21: r=0.761, P<0.001; TMPRSS3: r=0.727, P<0.001), a moderate negative correlation of TSPAN12 and BEX1 gene expression with adenoma diameter was observed (TSPAN12: r=-0.572, P=0.001; BEX1: r=-0.501, P=0.007) (Table 2).

**BEX1 Gene Expression is Increased in Micro-APAs and Aldosterone-Producing Micronodules**

In the expanded cohort of APAs (n=71), BEX1 gene expression was 2.76-fold higher in micro-APAs relative to macro-APAs (P<0.001), and 2.31-fold upregulated in APAs with NMD compared with APAs with a KCNJ5 mutation (P<0.0001) (Figure 2A, 2B). The linear negative correlation of BEX1 gene expression with adenoma diameter in the NMD subgroup is shown in Figure 2C. Micro-APAs (n=5) displayed a 3.85-fold increase in BEX1 expression compared with their paired adjacent cortex (P<0.05); whereas this difference was not observed for adrenals with macro-APAs (n=12) (Figure 2D). An analysis of BEX1 mRNA levels in other adrenal tumors showed no apparent differences in BEX1 gene expression according to tumor diameter in cortisol-producing adenomas (n=20) and in incidentalomas (n=8) (Figure 2E, 2F).

Analysis of publicly available transcriptome data demonstrated significantly higher BEX1 gene expression levels in aldosterone-producing micronodules (APMs, n=4), compared with paired zona fasciculata (zF, n=4; 8.75-fold, P<0.0001) and zona reticularis (n=4; 2.30-fold, P<0.05). In this small sample set, BEX1 expression was higher, but did not reach statistical
significance, in APMs versus paired adjacent zona glomerulosa (zG, n=4; 1.87-fold, \( P=0.0501 \)). Conversely, \( BEX1 \) gene expression was significantly increased in zG relative to paired zF (4.69-fold, \( P<0.001 \)) (Figure S4).

**Role of \( BEX1 \) in Inhibition of Cell Death by Ferroptosis**

We generated a stable human adrenocortical HAC15 cell line expressing \( BEX1 \) with a C-terminal DYKDDDDK tag (BEX1-DDK) (Figure 3A). Immunofluorescence staining demonstrated the localization of BEX1-DDK in the nucleus and cytoplasm (Figure 3B). The proportion of cells in the G0/G1, S and G2/M phases of the cell cycle was indistinguishable between HAC15 control and BEX1-DDK cells (Figure 3C, 3D). The effect of 2 \( \mu \)M staurosporine (STS)- an inducer of apoptosis- was highly similar in the BEX1-DDK cell line and HAC15 control cells in a cell viability assay (Figure 3E). In contrast, treatment with 4 \( \mu \)M RSL3 (a ferroptosis inducer) caused notably less cell death in the HAC15 BEX15-DDK cell line compared with HAC15 control cells (Figure 4A, 4B). The protective effect of \( BEX1 \) against RSL3-induced cell death, was confirmed by flow cytometry measurements using PI (propidium iodide) fluorescence staining of dead cells (Figure 4A, 4B, 4D). Specificity of RSL3-induced cell death was demonstrated by ablation of this response in the presence of 10 \( \mu \)M liproxstatin-1 (Lip-1, a ferroptosis inhibitor) (Figure 4C, 4D).

**Discussion**

We identified genes with significantly altered expression levels between APAs of distinct sizes with a focus on genes with a potential role in cell death and proliferation. We selected \( BEX1 \), encoding brain expressed X-linked 1,\(^{27} \) for further study because the function of this gene in the adrenal cortex is unknown and differential expression levels of \( BEX1 \) in different subsets
of APAs have been previously reported.\textsuperscript{5} Using in vitro functional analyses employing flow cytometry of human adrenocortical cell lines with stable overexpression of BEX1, we demonstrated a novel role for BEX1 as a suppressor of cell death by ferroptosis.

BEX1 transcripts are abundantly expressed in brain. In peripheral tissues, the highest gene expression levels are observed in the adrenal and testis.\textsuperscript{27, 28} Previous studies have reported a role for BEX1 in the regeneration of neurons,\textsuperscript{29} skeletal muscle\textsuperscript{30} and liver,\textsuperscript{31} associated with its function in cell cycle regulation\textsuperscript{29} and apoptosis.\textsuperscript{31} In addition, BEX1 has been identified as a part of a ribonucleoprotein processing complex that promotes translocation and maturation of mRNAs encoding pro-inflammatory genes in the heart.\textsuperscript{32}

Ferroptosis is an iron-dependent form of regulated cell death, morphologically and biochemically distinct from apoptosis, characterized by the accumulation of redox-active iron, lipid hydroperoxides, and oxidized polyunsaturated fatty acid-containing phospholipids.\textsuperscript{33-35} Adrenocortical cells are especially sensitive to ferroptosis,\textsuperscript{36, 37} an observation likely related to the vulnerability of steroidogenic tissues to redox imbalance caused by electron leakage by cytochrome P450 enzymes and reactive oxygen species generation.\textsuperscript{38-41}

Herein, we report an inverse correlation of BEX1 gene expression with APA diameter and an upregulation of BEX1 transcription in micro-APAs, but not macro-APAs, compared with paired adjacent adrenal cortex. The differential expression levels of BEX1 in different adrenal tissue samples may reflect different levels of steroidogenesis and production of reactive oxygen species. In this context, BEX1-mediated protection from ferroptosis may involve a response to increased steroidogenesis and oxidative stress thereby providing a growth advantage for zona
glomerulosa cells with aldosterone overproduction over adjacent cells. Thus, these findings may translate to a role for BEX1 in APA pathogenesis via promoting cell survival and facilitating adenoma formation. In support of this concept, analysis of publicly available transcriptome data\textsuperscript{26} revealed relatively high BEX1 gene expression in APMs (the revised nomenclature for aldosterone-producing cell clusters),\textsuperscript{23} a potential origin of APAs,\textsuperscript{42, 43} compared with paired adrenocortical zones.

Like APMs, micro-APAs display strong CYP11B2 (aldosterone synthase) immunostaining.\textsuperscript{23, 26, 44} In micro-APAs, CYP11B2 immunoreactivity per tumor area is more intense than in macro-APAs\textsuperscript{44} and the intensity of CYP11B2 immunostaining is inversely correlated with APA diameter.\textsuperscript{44, 45} The higher CYP11B2 expression associated with micro-APAs is likely required for sufficient aldosterone production to cause clinically overt PA from small adenomas.\textsuperscript{44} In support of this, in the present study, the baseline median plasma aldosterone concentration of the micro-APA group was similar to that of the macro-APA group, indicating the microadenomas could sustain high levels of steroidogenesis despite their smaller size.

If CYP11B2 immunoreactivity is taken as a surrogate of pathological steroidogenesis associated with an APA, decreased CYP11B2 immunoreactivity per tumor area with increasing APA diameter may suggest a reduced ability to elicit an oxidative stress response.\textsuperscript{39, 41, 46} Thus there would be a decreased requirement for anti-ferroptotic mechanisms and BEX1 gene expression. The protection from a cell death mechanism in tumors of a small size may appear paradoxical, but we have previously reported that APAs with NMD (unlike in APAs with a KCNJ5 mutation) display a decreased proliferation index with increasing adenoma diameter\textsuperscript{16} indicating a progressive activation of anti-proliferation mechanisms. Taken together,
mechanisms that control different forms of cell death and proliferation likely initiate and self-regulate tumor growth to restrict the size of a subset of APAs.

In a microarray analysis of 8 APAs with KCNJ5 mutations compared with 5 APAs with CACNA1D or ATP1A1 mutations, Azizan et al.\textsuperscript{5} identified BEX1 as a differentially expressed gene with significantly increased expression in adenosomas with CACNA1D or ATP1A1 mutations. Because these tumors tend to be small, with diameters less than 10 mm,\textsuperscript{5} our findings of increased BEX1 transcription in micro-APAs are in agreement with the report of Azizan et al.\textsuperscript{5} However, it is unclear if the high BEX1 expression and modulation of ferroptosis in the NMD group we detected is related to APAs of small diameter in general or to a specific aldosterone-driver mutation or mutations which we were unable to detect by our sequencing approach. In a later study,\textsuperscript{47} and of high relevance to the present work, the same group of researchers, identified a role for oxidative stress in APA pathogenesis by the analysis of the transcriptomes of APAs with their paired zona glomerulosa.\textsuperscript{47} The study demonstrated that the top canonical biological pathway associated with the differentially expressed genes (APA versus paired zona glomerulosa) was the NRF2 (nuclear factor erythroid 2–related factor 2)-mediated oxidative stress response\textsuperscript{47} which is a critical cellular mechanism to maintain intracellular redox homeostasis and limit oxidative damage.\textsuperscript{48}

A strength of our study is the transcriptome analysis using sample stratification by adenoma diameter and genotype to specifically identify genes that function in cell death and proliferation. An additional strength is the validation of gene expression levels in a large sample cohort from two expert referral centers using standardized diagnostic procedures.\textsuperscript{49} Furthermore, all APAs used in the study were resected from patients with complete
biochemical success after surgery highlighting the appropriate diagnosis of unilateral PA.\textsuperscript{2}

Finally, we performed a functional characterization of the \textit{BEX1} gene to identify a novel role in adrenocortical cells which is relevant to APA pathogenesis. The sequencing approach we used is a study limitation as it was not targeted to CYP11B2-positive lesions and therefore the NMD group potentially contained aldosterone-driver mutations.\textsuperscript{10-12}

In conclusion, the \textit{BEX1} gene is differentially expressed in APAs according to nodule diameter and protects human adrenocortical cells in vitro from a form of regulated cell death called ferroptosis.

\textbf{Perspectives}

Ferroptosis, a form of cell death associated with cell metabolism and redox biology, may function in the pathogenesis of APAs. Future studies are required to clarify this function and elucidate the potential role of BEX1. We are currently planning further transcriptomics studies using our stable BEX1 adrenocortical cell lines and flow cytometry analyses of single cell suspensions, isolated from APA and paired adjacent cortex tissue samples, treated with ferroptosis inducers and inhibitors to gain a better understanding of the role of BEX1 and of ferroptosis in adrenocortical cells.

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Conflicts of interest/ Disclosures

None
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Novelty and Significance

What is New?

• Transcriptome profiles of aldosterone-producing adenomas with highly diverse diameters are distinctly different
• Gene ontology enrichment analysis identifies over-representation of cell death in a subset of aldosterone-producing adenomas
• The BEX1 (brain expressed X-linked 1) gene is upregulated in micro- compared with macro-aldosterone-producing adenomas and with paired adjacent adrenal cortex
• In human adrenocortical cells in vitro, BEX1 confers protection from ferroptosis- a form of nonapoptotic regulated cell death

What is relevant?

• mRNA-seq analysis of aldosterone-producing adenomas of diverse sizes identifies a multitude of genes involved in cell growth mechanisms
• BEX1 may promote cell survival in small aldosterone-producing adenomas by mediating the inhibition of ferroptosis

Summary

BEX1 suppresses ferroptosis in human adrenocortical cells and may play a role in the pathogenesis of aldosterone-producing adenomas.
Figure 1. Distinct transcriptome profiles in APAs with different genotypes and adenoma diameters.

The heat map shows unsupervised hierarchical clustering of 500 genes with the largest coefficient of variation based on normalized FPKM (fragments per kilobase of transcript, per million mapped reads) identified by mRNA-seq (A). Row Z-score indicates the difference in expression level of a gene in standard deviation units from the mean expression level in all
samples. Volcano plots by mRNA-seq analysis highlight genes in red that were differentially expressed in APAs with NMD (B) or with a KCNJ5 mutation (C). Differential expression was defined as an adjusted $p$ value <0.05 using the Benjamini-Hochberg False Discovery Rate method. Red dots in the region of Log$_2$(fold change) <0 represent downregulated genes in APAs ≥30mm diameter compared with ≤10mm diameter; red dots in the region of Log$_2$(fold change) >0 represents upregulated genes in the NMD and $KCNJ5^{mut}$ subgroups as indicated. The second heatmap shows the mRNA expression levels of 10 genes, determined by real-time qPCR, of an expanded cohort of 71 APAs. The 10 genes selected for study were all DEGs of interest identified from the mRNA-seq analysis with a previously described role in cell death and proliferation (D). Genes studied are shown on the left of the figure, sample identity is color-coded at the top of the figure, and fold changes in gene expression (real-time qPCR quantification) are shown on the right, in the overall group (NMD+$KCNJ5^{mut}$) and stratified for APAs with NMD or $KCNJ5$ mutations ($KCNJ5^{mut}$). The color-coded matrix shows the relative quantification (RQ) values of genes for each sample ($2^{-\Delta\Delta Ct}$) compared with the median micro-APA gene expression level of each gene. RQ values were then transformed to a 0 to 100 grading scale with the smallest RQ value set as 0 and largest value as 100. Macro-APAs are defined as ≥10 mm; micro-APAs as <10mm diameter. APAs, aldosterone-producing adenomas; diam., diameter; $KCNJ5$, gene encoding potassium inwardly rectifying channel subfamily J member 5; $KCNJ5^{mut}$, APAs with $KCNJ5$ mutations; NMD, APAs with no mutation detected.
Figure 2. *BEX1* gene expression in adrenal tumors.

Real time qPCR analysis of *BEX1* gene expression in micro and macro-APAs (A) and in APAs with NMD and with a *KCNJ5*-mutation (B). A linear negative correlation of *BEX1* gene expression was observed with APA diameter in APAs with NMD (C). *BEX1* gene expression was higher in micro-APAs compared with APA adjacent cortex but not in macro-APAs relative to paired adjacent adrenal cortex (D). There were no apparent differences in *BEX1* gene expression according to adenoma size in cortisol-producing adenomas (E) and incidentalomas (F). Statistical analyses were performed on 2^{-ΔΔCt} values using a Mann-Whitney test, Pearson correlation analysis or a Wilcoxon matched-pairs signed rank test. *P*<0.05, ***P*<0.001, ****P*<0.0001. Each point represents a single sample. Horizontal lines indicate the median, whiskers represent 95% confidential intervals. APA, aldosterone-producing adenoma; *KCNJ5*, gene encoding potassium inwardly rectifying channel subfamily J member 5; *KCNJ5*mut, APAs with *KCNJ5* mutations; *n*, number; NMD, APAs with no mutation detected.
Figure 3. BEX1 has no effect on cell cycle progression and apoptosis in adrenocortical cells.

Western blot analysis of HAC15 cells selected for stable expression of empty vector (HAC15 Ctrl) or BEX1 with a C-terminal DYKDDDDK tag (BEX1-DDK) (A). Anti-DDDDK immunofluorescence staining of BEX1-DDK expressed in HAC15 cells compared with HAC15 Ctrl cells with F-actin and DAPI stain highlighting the cytoplasm and nucleus, respectively (B). HAC15 cell cycle analysis of Ctrl and BEX1-DDK cells, measured by flow cytometry with Vybrant DyeCycle violet DNA staining in a representative experiment (C) and from 3 independent experiments (D). Cell viability of HAC15 Ctrl and BEX1-DDK cells after 6 hours treatment with vehicle (0.02% DMSO) or 2 µM STS (staurosporine, inducer of apoptosis). Data are normalized to the HAC15 Ctrl cell line treated with 0.02% DMSO vehicle and data are shown from 3 independent experiments (E). In panel E, **difference (P<0.01) from HAC15 Ctrl cells treated with vehicle, ††difference (P<0.01) from HAC15 BEX1-DDK cells treated with vehicle. BEX1-DDK, HAC15 cells with stable expression of BEX1 with a C-terminal DYKDDDDK tag; Ctrl, HAC15 cells with stable expression of empty vector; DAPI, 4’, 6-diamidino-2-phenylindole, dihydrochloride; DDK, DDDDK tag; DMSO, dimethyl sulfoxide; F-actin, filamentous actin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; STS, staurosporine. Scale bar=50 µm.
Figure 4. BEX1 inhibits cell death by ferroptosis in adrenocortical cells.

HAC15 BEX1-DDK cells are less susceptible to cell death caused by the ferroptosis inducer RSL3 ([1S,3R]-RSL3) compared with the HAC15 Ctrl cell line (upper panels of A, B). Analysis of cell death by flow cytometry with PI (propidium iodide) of HAC15 Ctrl and BEX1-DDK cells after treatment with vehicle (0.004% DMSO), 4 µM RSL3 or 4 µM RSL3 + 10 µM Lip-1 for 24 hours. The proportion (%) of PI-negative cells (alive, in green) or positive cells (dead, in red) measured by flow cytometry is indicated within each chromatogram in a representative experiment (lower panels of A, B, C) and from 3 independent experiments (D). Bars represent means of three independent experiments, error bars indicate SEM. P values were calculated by two-way ANOVA with a Bonferroni’s post-test. In panel D, **difference (P<0.01) between HAC15 BEX1-DDK cells and HAC15 Ctrl cells treated with RSL3. BEX1-DDK, HAC15 cells with stable expression of BEX1 with a C-terminal DYKDDDDK tag; Ctrl, HAC15 cells with stable expression of empty vector; DDK, DDDDK tag; DMSO, dimethyl sulfoxide; Lip-1, liproxstatin-1; PI, propidium iodide; RSL3, (1S,3R)-RSL-3.
<table>
<thead>
<tr>
<th>Variables</th>
<th>Total cohort</th>
<th>Macro-APA</th>
<th>Micro-APA</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=71)</td>
<td>(n=58)</td>
<td>(n=13)</td>
<td></td>
</tr>
<tr>
<td>Age at surgery (years; n=71)</td>
<td>48.2 ± 11.5</td>
<td>48.2 ± 11.4</td>
<td>48.1 ± 12.4</td>
<td>0.978</td>
</tr>
<tr>
<td>Sex (ref. women; n=71)</td>
<td>41 (57.7%)</td>
<td>37 (63.8%)</td>
<td>4 (30.8%)</td>
<td>0.029</td>
</tr>
<tr>
<td>BMI (kg/m²; n=66)</td>
<td>26.2 [22.7-30.1]</td>
<td>26.0 [22.4-30.0]</td>
<td>26.4 [23.4-31.5]</td>
<td>0.502</td>
</tr>
<tr>
<td>Systolic BP (mmHg; n=68)</td>
<td>150 [140-166]</td>
<td>149 [140-161]</td>
<td>160 [150-174]</td>
<td>0.029</td>
</tr>
<tr>
<td>Diastolic BP (mmHg; n=68)</td>
<td>94 [85-101]</td>
<td>92 [85-100]</td>
<td>100 [88-108]</td>
<td>0.217</td>
</tr>
<tr>
<td>Duration HTN (months; n=69)</td>
<td>97 [29-171]</td>
<td>99 [24-174]</td>
<td>97 [51-207]</td>
<td>0.586</td>
</tr>
<tr>
<td>Anti-HTN meds (DDD; n=66)</td>
<td>3.0 [1.5-4.8]</td>
<td>3.0 [1.1-4.5]</td>
<td>3.7 [2.6-4.9]</td>
<td>0.179</td>
</tr>
<tr>
<td>PAC (pmol/L; n=69)</td>
<td>838 [590-1419]</td>
<td>859 [586-1404]</td>
<td>805 [571-1523]</td>
<td>0.939</td>
</tr>
<tr>
<td>DRC (mU/L; n=36)</td>
<td>3.4 [2.0-7.6]</td>
<td>3.3 [2.0-8.2]</td>
<td>4.5 [2.4-6.5]</td>
<td>0.903</td>
</tr>
<tr>
<td>ARR_DRC (n=36)</td>
<td>197 [92-356]</td>
<td>218 [92-371]</td>
<td>160 [89-283]</td>
<td>0.480</td>
</tr>
<tr>
<td>PRA (pmol/L/min; n=32)</td>
<td>2.6 [2.3-6.4]</td>
<td>2.6 [2.6-5.1]</td>
<td>6.4 [1.3-8.3]</td>
<td>0.564</td>
</tr>
<tr>
<td>ARR_PRA (n=32)</td>
<td>352 [142-596]</td>
<td>377 [150-609]</td>
<td>225 [111-841]</td>
<td>0.458</td>
</tr>
<tr>
<td>Lowest serum K⁺ (mmol/L; n=70)</td>
<td>3.0 ± 0.6</td>
<td>2.9 ± 0.6</td>
<td>3.2 ± 0.4</td>
<td>0.145</td>
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<tr>
<td>Nodule diam. (mm; n=71)</td>
<td>15.0 [11.0-21.0]</td>
<td>16.0 [14.0-22.8]</td>
<td>7.0 [6.5-8.0]</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>APAs_KCNJ5 mutation (n=43)</td>
<td>43 (60.6%)</td>
<td>39 (67.2%)</td>
<td>4 (30.8%)</td>
<td>0.015</td>
</tr>
<tr>
<td>APAs_NMD (n=28)</td>
<td>28 (39.4%)</td>
<td>19 (32.8%)</td>
<td>9 (69.2%)</td>
<td>0.015</td>
</tr>
<tr>
<td><strong>Clinical outcome (n=63)</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.115</td>
</tr>
<tr>
<td>Complete success</td>
<td>27 (42.9%)</td>
<td>25 (47.2%)</td>
<td>2 (20.0%)</td>
<td></td>
</tr>
<tr>
<td>Partial success</td>
<td>28 (44.4%)</td>
<td>23 (43.4%)</td>
<td>5 (50.0%)</td>
<td></td>
</tr>
<tr>
<td>Absent success</td>
<td>8 (12.7%)</td>
<td>5 (9.4%)</td>
<td>3 (30.0%)</td>
<td></td>
</tr>
<tr>
<td><strong>Biochemical outcome (n=61)</strong></td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
</tr>
</tbody>
</table>
Clinical and biochemical parameters of patients with APA stratified by adenoma diameter.

All variables refer to baseline data. Nodule diam. refers to the diameter of the largest adrenal nodule at pathology. Macro-APAs were defined by diameter of the largest nodule ≥10 mm; micro-APAs were defined by diameter of the largest nodule <10 mm. Quantitative normally distributed variables are expressed as means ±SD and quantitative non-normally distributed variables are reported as medians [IQR]. Categorical variables are presented as absolute numbers and percentages. P values were calculated using Chi-square and Fisher’s exact tests or t test or Mann-Whitney test as appropriate. P values of less than 0.05 were considered significant. APA, aldosterone-producing adenoma; ARR, aldosterone-to-renin ratio; BMI, body mass index; BP, blood pressure; DDD, defined daily dose; diam., diameter; DRC, direct renin concentration; HTN, hypertension; K⁺, potassium ions; KCNJ5, gene encoding potassium inwardly rectifying channel subfamily J member 5; meds, medications; n, number; NA, not applicable; NMD, no mutation detected; PAC, plasma aldosterone concentration; PRA, plasma renin activity; ref., reference. The defined daily dose is the assumed average maintenance dose per day for a drug used for its main indication in adults according to ATC/DDD Index 2019 https://www.whocc.no/atc_ddd_index/.

<p>| Complete success | 61 (100.0%) | 50 (100.0%) | 11 (100.0%) |
| Partial success  | 0 (0.0%)    | 0 (0.0%)    | 0 (0.0%)    |
| Absent success   | 0 (0.0%)    | 0 (0.0%)    | 0 (0.0%)    |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Overall</th>
<th>NMD</th>
<th>KCNJ5mut</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>r</td>
<td>P</td>
<td>r</td>
</tr>
<tr>
<td>TSPAN12</td>
<td>Tetraspanin 12</td>
<td>-0.388</td>
<td>0.001</td>
<td>-0.572</td>
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<tr>
<td>SFRP2</td>
<td>Secreted frizzled-related protein 2</td>
<td>-0.038</td>
<td>0.760</td>
<td>-0.397</td>
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<tr>
<td>BAI1</td>
<td>Brain-specific angiogenesis inhibitor 1</td>
<td>-0.105</td>
<td>0.411</td>
<td>-0.210</td>
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<tr>
<td>BEX1</td>
<td>Brain expressed X-linked 1</td>
<td>-0.376</td>
<td>0.001</td>
<td>-0.501</td>
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<tr>
<td>CCL21</td>
<td>Chemokine, C-C motif, ligand 21</td>
<td>-0.152</td>
<td>0.207</td>
<td>-0.249</td>
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<td>DKK1</td>
<td>Dickkopf-related protein 1</td>
<td>-0.139</td>
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<td>FBXL21</td>
<td>F-box and leucine rich repeat protein 21</td>
<td>0.361</td>
<td>0.005</td>
<td>0.761</td>
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<tr>
<td>TFPI2</td>
<td>Tissue factor pathway inhibitor 2</td>
<td>0.039</td>
<td>0.748</td>
<td>0.017</td>
</tr>
<tr>
<td>TMRPSS3</td>
<td>Transmembrane serine protease 3</td>
<td>0.443</td>
<td>0.001</td>
<td>0.727</td>
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<tr>
<td>BMP4</td>
<td>Bone morphogenetic protein 4</td>
<td>0.111</td>
<td>0.358</td>
<td>0.389</td>
</tr>
</tbody>
</table>

**Table 2. Correlation of gene expression levels with APA diameter according to genotype.**

Values indicate Pearson correlation coefficients (r) and respective P values in the overall group (NMD+KCNJ5mut) of APAs or stratified for APAs with NMD or KCNJ5 mutations. Gene expression levels of 71 APAs (28 with NMD and 43 with KCNJ5 mutations) were determined.
by real-time qPCR as described in the online supplemental methods section. *KCNJ5*, gene encoding potassium inwardly rectifying channel subfamily J member 5; *KCNJ5*<sup>mut</sup>, *KCNJ5* gene mutations; NMD, no mutation detected.