Table S1: Clinical and biological characteristics of patients analyzed in the study.

Patient	R337H mutation	Age (months)	Gender	Size (cm^3)	Weight (g)	Symptoms	Sandrini´s classification	Metastasis	Chemotherapy	Relapse	Event (dead or relapse)	Outcome	Overall survival (months)	Event free survival (months)
7	yes	22	female	6,28	10	Mixed	1	No	No	No	No	Alive	173	173
54	yes	101	female	527,18	580	Mixed	2	No	No	Yes	Yes	Dead	20	20
58	yes	27	male	78,81	80	Mixed	1	No	No	No	No	Alive	274	274
105	yes	11	male	41,19	25,9	Mixed	1	No	No	No	No	Alive	101	101
148	yes	20	male	36,24	60	Mixed	2	No	No	Yes	Yes	Alive	152	7
150	no	137	female	1725,9	2350	No symptoms	4	yes	Yes	Yes	Yes	Alive	218	53
164	yes	95	female	191,29	230	Virilizing	4	yes	Yes	Yes	Yes	Dead	15	7
171	yes	92	female	284,51	380	Mixed	1	yes	Yes	Yes	Yes	Dead	29	9
195	no	13	male	28,11	40	Mixed	1	No	No	No	No	Alive	206	206
209	no	85	female	0,94	3,3	Mixed	2	No	No	No	No	Alive	202	202
235	yes	28	female	423,63	377	Virilizing	1	No	Yes	No	No	Alive	262	262
361	yes	36	female	14,06	16	Mixed	1	No	No	No	No	Alive	177	177
383	yes	18	female	39,23	35	Mixed	4	No	No	No	No	Alive	246	246
387	yes	185	male	585,76	703	Cushing	4	No	No	No	Yes	Dead	8	8
525	yes	33	male	1059,08	828	Cushing	1	yes	Yes	Yes	Yes	Dead	7	5
545	yes	34	male	26,65	28,5	Mixed	1	No	No	No	No	Alive	227	227
686	yes	22	female	16,57	20	Mixed	1	No	No	No	No	Alive	16	16
698	no	145	female	82,37	53	Cushing	4	No	No	No	No	Alive	174	174
799	yes	66	female	376,56	351	Virilizing	1	yes	Yes	Yes	Yes	Dead	15	6
922	yes	23	female	11,71	39	Mixed	2	No	No	No	No	Alive	174	174
950	yes	5	female	91,79	153	Mixed	1	No	No	No	No	Alive	176	176
1116	no	40	female	18,83	15	Mixed	2	No	No	No	No	Alive	163	163
1340	yes	82	male	59,62	108	Virilizing	2	No	No	Yes	Yes	Dead	7	4
1393	yes	21	female	56,48	84	Mixed	1	No	No	No	No	Alive	11	11
1455	yes	18	female	125,52	151	Mixed	1	No	No	No	No	Alive	120	120
1525	yes	34	female	3,77	9	Virilizing	1	No	No	No	No	Alive	131	131
1993	yes	21	female	36,61	41	Mixed	1	No	No	No	No	Alive	101	101
2049	yes	15	female	15	NA	Virilizing	1	No	No	No	No	Alive	84	84
2329	no	157	female	16,11	154	No symptoms	1	No	No	No	No	Alive	83	83
2451	yes	15	male	36,59	50	Mixed	1	No	No	No	No	Alive	58	58
2461	yes	18	female	17,57	19	Virilizing	1	No	No	No	No	Alive	65	65
2575	yes	9	female	11,13	9	No symptoms	2	No	No	No	No	Alive	53	53
2637	yes	25	female	16,24	71	Virilizing	1	No	No	No	No	Alive	54	54
2711	yes	35	female	520,12	402	Virilizing	2	No	No	No	No	Alive	39	39
5705	yes	13	female	47,07	35	Mixed	1	No	No	No	No	Alive	133	133
5908	yes	12	male	109,83	102	Mixed	1	No	No	No	No	Alive	66	66
8202	yes	29	female	49,57	80	Mixed	1	No	No	Yes	Yes	Dead	16	3
9600	yes	17	female	25,52	20	Mixed	1	No	No	No	No	Alive	186	186
14606	yes	18	female	56,01	50	Virilizing	4	No	No	No	No	Alive	36	36
16007	yes	16	female	15,08	16	Mixed	1	No	No	No	No	Alive	99	99
17903	no	21	male	196,58	75	No symptoms	2	yes	Yes	No	No	Alive	153	153
23407	yes	12	female	17,79	40	Mixed	4	No	No	No	No	Alive	113	113
26406	yes	92	female	73,22	102	Mixed	1	No	No	No	No	Alive	125	125
29091	yes	14	male	112,21	7	Virilazing	1	yes	Yes	Yes	Yes	Alive	166	10
32804	yes	10	female	40,27	50	Mixed	2	No	No	No	No	Alive	147	147
35104	yes	29	female	0,23	40	Mixed	2	No	No	No	No	Alive	50	50
38701	yes	16	female	192,82	150	Virilazing	1	No	No	No	Yes	Dead	0	0
39499	yes	111	female	527,18	520	Virilazing	2	No	Yes	Yes	Yes	Dead	9	3
40007	yes	15	female	12	70	Mixed	2	No	No	No	No	Alive	109	109
41203	yes	187	female	596,22	634	Mixed	1	No	No	No	No	Alive	133	133
41503	no	17	female	344,2	395	Virilazing	3	No	No	No	No	Alive	140	140
44608	yes	22	female	52,3	84	Virilazing	3	No	No	Yes	Yes	Alive	28	23
47009	yes	16	female	58,25	68	Mixed	1	No	Yes	No	No	Alive	80	80

48705	yes	19	female	456,67	228	Virilazing	1	No	Yes	No	No	Alive	130	130
48708	yes	13	female	28,87	25	Mixed	2	No	Yes	No	No	Alive	94	94
53201	yes	17	female	13,71	15	Mixed	4	No	Yes	Yes	Yes	Alive	179	3
59603	yes	12	female	13,69	15	Mixed	1	No	No	No	No	Alive	120	120
61301	no	13	female	313,8	340	Mixed	1	No	No	No	No	Alive	172	172
63205	yes	19	female	313,8	340	Virilazing	1	yes	Yes	No	Yes	Dead	7	7
68705	yes	25	female	39,43	65	Mixed	3	No	No	No	No	Alive	123	123
70203	yes	10	female	15,32	35	Mixed	1	No	No	No	No	Alive	153	153
74099	yes	44	female	40,27	20	Mixed	1	No	No	No	No	Alive	203	203
74804	yes	52	male	621,75	810	Mixed	1	No	Yes	No	No	Alive	143	143
76202	yes	5	male	17,42	20	Virilazing	1	No	No	No	No	Alive	148	148
81108	yes	38	female	0,45	6	Mixed	2	No	No	No	No	Alive	90	90
84001	yes	64	female	34,29	55	Mixed	1	No	No	No	No	Alive	174	174

Table S2: Primers sequences used to evaluate gene expression of the *PRKAB2, TSC1, STK11*, and *GUS* genes.

Gene	Primer sequence
PRKAB2	Forward: 5′AAATCCCCACCCATCCTTC3
	Reverse: 3′GCATAACATGGTTGGGCTCA5
TSC1	Foward:5′AGCCAATGATGGAGCATGTG3′
	Reverse: 3′ GGCACACTCGATCACAACAT5′
STK11	Foward: 5′CTTCTTTGGCGACGAGTCAA3′
	Reverse: 3′ATGATGGGATCTGTCGAGG5
GUS	Foward: 5′GAAAATATGTGGTTGGAGAGCTCATT3′
	Reverse: 3′CCGAGTAAGATCCCCTTTTTA5′

Methods (Supplemental material)

Cell line

The NCI-H295R [H295R] adrenocortical carcinoma cell line (ACTC® CRL-2128™), was cultured in DMEM-F12 medium supplemented with 2.5% NuSerum (CORNING, Teo Oak Park, Bedford, MA, USA), 1% ITS (GIBCO, Grand Island, NY 14072, USA), 60 mg/mL penicillin, and 100 µg/mL streptomycin (GIBICO, Grand Island, NY 14072, USA) in humid atmosphere containing 5% CO_2, at 37 °C. An STR test was performed to confirm lineage authenticity. The H295R cell line present an inactivation mutation in the ***TP53*** gene and an activating mutation in the ***CTNNB1*** gene (c.T133C:p.S45P). These cells are the only commercially available cell line that was confirmed as a human ACT.

Treatment with Rottlerin

NCI-H295R cells were treated with Rottlerin (protein kinase C delta inhibitor: 1610, Tocris Bioscience, Atlantic Road, Bristol) for 6-72h according to each specific assay. A 1 mmol/L Rottlerin stock solution was prepared in dimethylsulfoxide (DMSO), and aliquots of this solution were stored at -20 °C. The IC_{50} and IC_{25} values were established by using the Compusyn software (ComboSyn Inc.)

RNA extraction and gene expression by real-time PCR (RT-qPCR)

ACT,non-neoplastic adrenal and NCI-H295R samples were subjected to total RNA extraction by using the TRIZOL® reagent (InvitrogenInc, Carlsdab, CA, USA). cDNA was synthesized with the High Capacity® kit (AppliedBiosystems, Foster City, CA, USA) according to the manufacturer. For gene expression analysis, NCI-H295R cells were treated with Rottlerin for 48h, followed by RNA extraction and cDNA synthesis by using the same protocol mentioned above. Gene expression was analyzed by the qRT-PCR technique. The *GUSB* gene was used as an endogenous control, and the mean gene expression values of six non-neoplastic adrenal samples were used as calibrator. The employed primers are shown in Supplementary Table 1. Experiments were carried out on the QuantStudio™ 12K Flex equipment (Applied Biosystems, Foster City, CA, USA), by using the SYBR Green® and GoTaq Probe® PCR Master Mix (Applied Biosystems, Foster City, CA, USA): *GUSB* (4326320E), *STAR* (00986559), *MYCN* (00232074), *TCF7L2* (Hs01009044), *LEF1* (Hs01547250), *AXIN2*

(00610344), *GSK3B* (Hs01047719), and *AKT1* (00178289). Relative gene expression was quantified with the $2^{-\Delta\Delta CT}$ equation (Livak & Schmittgen, 2001). The standard curve for each primers was constructed to determine efficiency, by considering an efficiency of 90–100% (slopes between -3.6 and -3.3). All the samples were analyzed in at least three independent experiments performed in triplicate.

Cell viability assay

Cell viability was analyzed by using the CellTiter Glo® Cell Luminescent Cell Viability kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, NCI-H295R cells were cultivated in a 96-well plate at a density of 2×10^4 cells/well; and treated with 4.5 µM (IC_{50}) or 2.25 µM (IC_{25}) of Rottlerin for 24, 48, or 72 h. After treatment, the luminescence was measured using a SpectraMax® L. At least three independent experiments were performed for each concentration and time in triplicate.

Clonogenic Assay

NCI-H295R cells were treated with Rottlerin at 4.5 µM (IC_{50}) or 2.25 µM (IC_{25}) in 25-cm^3 bottles, at a confluence of 4 x 10^6, for 24 or 48 h. Then, cells previously treated with Rottlerin (2.5 x 10^2 cells/well) were seeded in a 12-well plate and cultured with normal medium for additional four weeks for colony formation. Medium was changed every 48 h. Colonies were then fixed with methanol,stained with Giemsa and counted. Only colonies with more than 50 cells were considered for analysis. The assays were analyzed in at least three independent experiments performed in triplicate.

Transwell Assay

NCI-H295R cells were treated with 4.5 µM (IC_{50}) or 2.25 µM (IC_{25}) of Rottlerin in 25-cm^3 bottles, at a confluence of 4 x 10^6, for 48 h. Then, 2 x 10^5 cells suspended in DMEM-F12 medium without supplement were seeded per insert. Supplemented medium was added to the 24-well plate, below the insert, to stimulate cell migration. Cells were allowed to migrate for 72 h and then fixed with methanol and stained with Giemsa 1% (diluted in phosphate buffer). The migrated cells were photographed under an inverted microscope in three randomly selected fields along the scraped line and measured by using the ImageJ software version 1.48 (NIH, Washington, USA). The assays were repeated in three independent experiments, in triplicate.

Hormone measurement

For hormone quantification, 5 x 10^5 NCI-H295R cells/well ware seeded in a six-well plate and after24 h were treated with DMSO or 4.5 µM (IC_{50}) or 2.25 µM (IC_{25}) of Rottlerin for 48 h. After that, the culture medium was collected and immediately stored at -80 °C. Cortisol, D4-androstenedione (D4), and testosterone concentrations were determined by radioimmunoassay (RIA) as previously described (DOI:10.1210/jcem.74.1.1309366). All the hormones were measured in triplicate and the mean was normalized to the cell viability effects in the same treatment conditions.

Western blotting

For western blotting analysis, NCI-H295R cells were treated with 4.5 µM (IC_{50}) or 2.25 µM (IC_{25}) of Rottlerin for 6, 12, 24, or 48 h, followed by protein extraction. between 20-40 µg of proteins was separated by SDS-PAGE electrophoresis and membrane incubated with 1:1000 anti-AMPKβ2 primary antibodies (4148S), 1:1000 p-AMPK (#2535), 1:1000 AMPKα-total (#2532), 1:1000 p-mTOR (#2971), and 1:1000 mTOR-total (#2983), obtained from Cell Signaling Technology, Danvers, MA, USA, and 1:2500 anti-GAPDH (sc-47724) and 1:500 MAP LC3B (sc-271625), obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). Anti-mouse IgG 1:5000 (#7076) and anti-rabbit IgG 1:5000 (#7074) secondary antibodies were obtained from Cell Signaling Technology, Danvers, MA, USA. A molecular weight marker (MW) and the protein of interest run on the same gel. The detection of the MW is done by colorimetry, just to control the size of the band of interest. The protein was detected by chemiluminescence using the ECL™ Western blotting Analysis System (Amersham GE Healthcare, Buckinghamshire, UK). Intensity was quantified by using the ImageJ software version 1.48 (NIH, Washington, USA), relativized by the control, and normalized by an endogenous protein (GAPDH). Membrane stripping was performed a maximum of three times using Restore Western Blot Stripping buffer (Thermo Scientific, Illinois, USA).

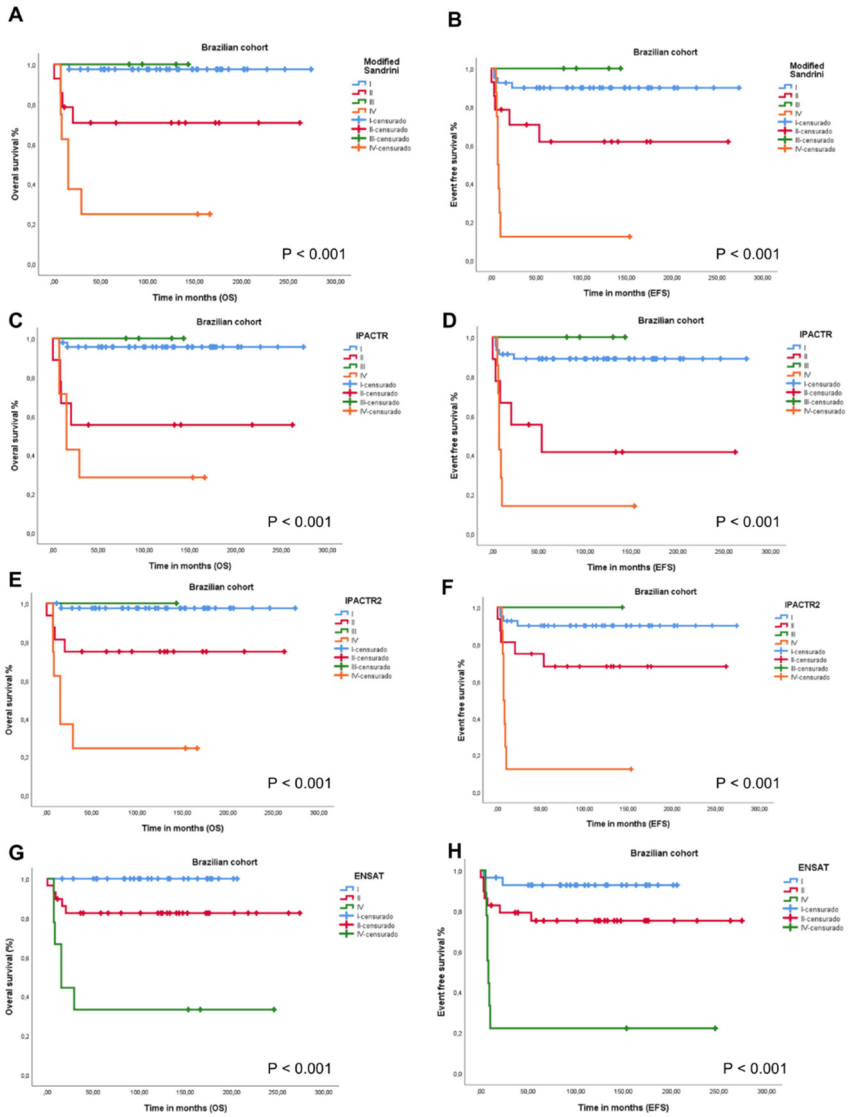
RNA sequencing (RNAseq)

To investigate the role played by Rottlerin in NCI-H295R cells, global gene expression was evaluated by RNAseq analysis in cells treated with Rottlerin (IC_{50}) or control (DMSO) for 48 hours. The Illumina Stranded mRNA Sample Preparation kit (Illumina Inc) was used to prepare the samples. The generated libraries were evaluated by using the Tapestation equipment (Agilent Technologies) and quantified on a Qubit fluorimeter with the Qubit dsDNA BR Assay kit (Life

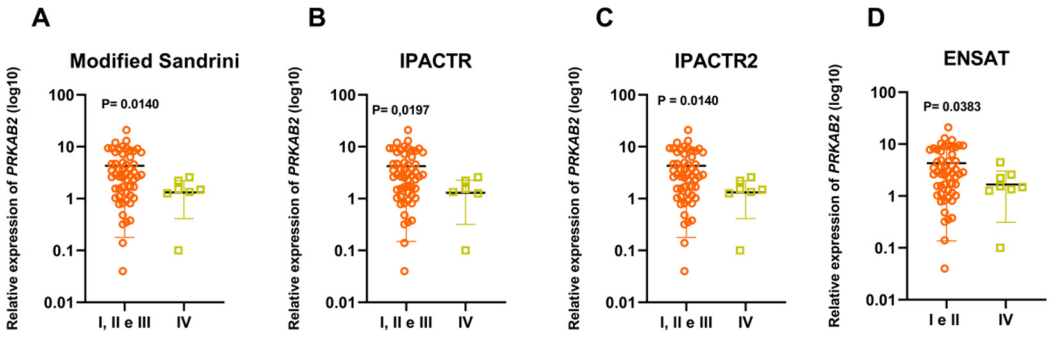
Technologies). Clustering and sequencing were performed on the Illumina NovaSeq 6000 equipment by using the NovaSeq SP Kit (200 cycles) (Illumina Inc), to generate 800 million reads per sequencing. The data quality of the two samples (Rottlerin at IC_{50} and DMSO) was evaluated by using the FastQC (0.11.3) and MultiQC (v1.10) programs, which provide information about basic sequence statistics, quality, GC content, sequence size distribution, sequence duplication levels, and over-represented sequences. For alignment, mapping, and counting of the sequences against the reference genome, the STAR aligner (v2.7.8a) was used. From the count of reads in each sample, the R programming language (v4.1.2) was applied; more specifically, the DESeq2 package was employed, which enabled differential expression between the different conditions of the experiment to be analyzed. On the basis of Log Fold-Change (LogFC), the differentially expressed genes (DEGs) were separated into high and low expression and subsequently enriched for deregulated pathways through the enrichment platform (https://maayanlab.cloud/Enrichr/). Sequencing was carried out at the Institute for Cancer Research (IPEC), Guarapuava – PR.

Statistical analysis

All data obtained from functional assays and patients' samples evaluations were analyzed by using the GraphPadPrism 8.0 (GraphPad Software, San Diego, CA, USA) or SPSS 20.0 (SPSS Inc. Chicago, USA) statistical programs. At least three independent experiments were carried out in triplicate and the average of the experiments was considered. The Mann-Whitney test was used to analyze patients' gene expression and clinical characteristics. The One-way and Two-way – ANOVA tests were used for functional tests, with a post-hoc Bonferroni test were used for functional; $p < 0.05$ was adopted as the level of statistical significance. Analysis of five-year event-free survival (with relapse and/or death from any cause being considered an unfavorable event) and five-year overall survival (with death being considered an unfavorable event) rates was based on Kaplan-Meier curves and log-rank test. The median *PRKAB2* expression was used as the cut-off point for high or low expression. The multivariate test using the Cox regression model was applied to assess independence of the factors in the predictive capacity.



Supplementary Figure S1. Kaplan-Meier curves and log-rank test show a lower survival in stage IV patients in our cohort. (A) OS ENSAT cohort (p <0.001) Stage I (n=28) II (n= 29), IV (n =9). (B) EFS ENSAT (p<0.001) stage I (n=28), II (n=29) and IV (n=9). (C) OS IPACTR stage I (n=46), II (n=9), III (n=4), and IV(n=7). (D) EFS IPACTR stage I (n= 46), II (n= 9), III (n= 4), and IV (n= 7). (E) OS IPACTR2 (p < 0.001), Stage I (n=41), II (n=26), III (n=1), and IV (n=8). (F) EFS IPACTR2 (p<0.001), stage I (n=41), II (n=16), III (n=1), and IV (n=8). (G) Modified Sandrini (p < 0.001) Stage I (n=40), II (n=14), III (n=4) and IV (n=8). (H) Modified EFS Sandrini (p< 0.001) stage I (n=40), II (n=14), III (n=4), and IV (n=8).



Supplementary Figure S2. (A) ENSAT patients in stages I, II, and III have lower PRKAB2 gene expression (p= 0.03); (B) IPACTR patients in stages I, II, and III showed lower expression of the PRKAB2 gene when compared to patients in stage IV (p=0.019); (C) IPACTR2 Patients with stage I, II and III showed lower expression of the PRKAB2 gene when compared to controls (p = 0.014); (D) Modified Sandrini, patients with stages I, II and III lower PRKAB2 gene expression when compared to stage IV (p= 0.0197) (Mann-Whitney test).