A Novel Patient-Derived Cell Line of Adrenocortical Carcinoma Shows a Pathogenic Role of Germline MUTYH Mutation and High Tumour Mutational Burden

Laura-Sophie Landwehr

University Hospital Würzburg <https://orcid.org/0000-0002-6315-4176>

Jochen Schreiner

University Hospital Würzburg

Silke Appenzeller

University of Würzburg

Stefan Kircher

University of Würzburg

Sabine Herterich

University Hospital Würzburg

Martin Fassnacht

University Hospital Würzburg

Matthias Kroiss

University Hospital Munich

Isabel Weigand (✉ isabel.weigand@med.uni-muenchen.de)

University Hospital Munich

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Abstract

Background

The response of advanced adrenocortical carcinoma (ACC) to current chemotherapies is unsatisfactory and a limited rate of response to immunotherapy was observed in clinical trials. High tumour mutational burden (TMB) and the presence of a specific DNA signature are characteristic features of tumours with mutations in the gene *MUTYH* encoding the mutY DNA glycosylase. Both have been shown to potentially predict the response to immunotherapy. High TMB in an ACC cell line model has not been reported yet.

Methods

The JIL-2266 cell line was established from a primary ACC tumour, comprehensively characterised and oxidative damage, caused by a dysfunctional mutY DNA glycosylase, confirmed.

Results

Here, we characterise the novel patient-derived ACC cell line JIL-2266, which is deficient in *MUTYH*-dependent DNA repair. JIL-2266 cells have a consistent STR marker profile that confirmed congruence with primary ACC tumour. Cells proliferate with a doubling time of 41 ± 13 hours. Immunohistochemistry revealed positivity for steroidogenic factor-1. Mass spectrometry did not demonstrate significant steroid hormone synthesis. JIL-2266 have hemizygous mutations in the tumour suppressor gene *TP53* (c.859G>T:p.E287X) and *MUTYH* (c.316C>T:p.R106W). Exome sequencing showed 683 single nucleotide variants and 4 insertions/deletions. We found increased oxidative DNA damage in the cell line and the corresponding primary tumour caused by impaired mutY DNA glycosylase function and accumulation of 8-oxoguanine.

Conclusion

This model will be valuable as a pre-clinical ACC cell model with high TMB and a tool to study oxidative DNA damage in the adrenal gland.

Background

Adrenocortical carcinoma (ACC) is a rare endocrine cancer affecting approximately 0.5 to 2 per million people annually [1]. About 60% of patients present symptoms of adrenal steroid excess, such as Cushing syndrome or virilisation [2]. Surgery is the only curative approach but many patients with ACC experience a relapse, even after complete surgical resection [3, 4]. Mitotane is the only approved drug in metastatic disease [5] and used both in a palliative and adjuvant setting [6, 7]. Objective response rate of advanced ACC is however low with only 20% [8] and severe adverse effects are common [2, 9]. Combination of etoposide, doxorubicin and cisplatin (EDP) is the most effective cytotoxic chemotherapy, but median overall survival is still poor with only 12-15 months [10].

Somatic mutations in the Wnt/ β -catenin pathway [11] are present in ~40 % of cases with *CTNNB1* mutations present in 25% [12] and *ZNRF3* mutations in 21% [13]. Inactivating mutations in the tumour suppressor *TP53* occur in at least another 20% of sporadic ACC [11]. Increased expression of *IGF2* (Insulin like growth factor 2) that is found in ~90% of ACC [14-16] provided the rationale for a phase III clinical trial with the selective small molecule IGF-1R inhibitor linsitinib but the results were disappointing [17]. Only few patients derive clinical benefit from multi-tyrosine kinase inhibitors that have been clinically tested [18].

Genetic predisposition to ACC includes Li-Fraumeni syndrome (LFS) caused by mutations in the tumour suppressor *TP53* in 3-7 % of ACC [19, 20]. Mutations in the mismatch repair (MMR) genes *MSH2*, *MSH6*, *MLH1*, *PMS2* cause Lynch syndrome (LS) and have been found in at least 3% of ACC [1, 21]. Impaired MMR protein function leads to high tumour mutational burden (TMB) and – consecutively - the presence of a high number of tumour specific neoantigens that may trigger an anti-tumoural immune response [22, 23].

While the overall response rate of ACC to immunotherapy in clinical trials was heterogeneous [24-26], 2/9 of ACC patients responding to the programmed cell death 1 (PD-1) inhibitor pembrolizumab in a clinical phase II trial had LS [27]. In a patient-derived mouse xenograft of a LS-associated ACC, experimental immune checkpoint inhibition led to increased antitumoural immune cell infiltration and consecutive treatment response [28].

Similar to LS-associated DNA repair deficiencies, inactivating mutations in the *MUTYH* gene involved in oxidative DNA damage repair confer a heritable predisposition to colorectal carcinoma termed *MUTYH*-associated polyposis. *MUTYH*-deficient tumours exhibit a high TMB and are responsive to immune checkpoint inhibition [29]. Two genome-wide studies identified *MUTYH* germline mutations in four ACC with high TMB [30].

Yet, cell culture models to investigate factors of response and resistance to T cell-mediated anti-tumoural response in ACC [31] are scarce. Until recently, H295R [32] cells first described in 1990 were the only available cell line. The first paediatric ACC patient-derived xenograft (PDX) model (SJ-ACC3) was reported by Pinto et al. [33] and in 2016, Hantel and colleagues successfully developed the first adult ACC PDX and established a respective cell line, termed MUC-1 [34]. The publication in 2018 of two additional PDX-derived ACC cell lines including one from a LS patient [35] increased the number of available human ACC cell lines to four, which now allows comparative studies that better reflect the genetic heterogeneity of ACC [16, 36-38].

Here, we present a newly established ACC cell line with high mutational burden, which was generated directly from a patient-derived tumour. Since the development of the H295R cell line 30 years ago, this is the first ACC cell line that was transferred directly to cell culture.

Materials And Methods

Patients

All patients included were participants of the ENSAT (European Network for the Study of Adrenal Tumours) registry and biobank. The study was approved by the Ethics Committee of the University of Würzburg (# 88/11) and all patients provided written informed consent for the use of tissue, cells, clinical data and genetic characterisation.

Establishment of the ACC tumour cell line

At the time of surgery, 1.3 mg of the primary tumour were used for cell culture after removal of the surrounding fat tissue. The tumour piece was minced and a single cell suspension was obtained using the gentleMACS Dissociator and Tumour Dissociation Kit (both Miltenyi Biotec), following the manufacturer's instructions. Culture medium was supplemented with 5 µmol/l Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Sigma-Aldrich), 10 µl/ml penicillin G/streptomycin (P/S, Sigma-Aldrich) and 250 µg/ml amphotericin B (Sigma-Aldrich) in adaption of a published protocol [39].

Cells were grown in 3:1 (v/v) Dulbecco's modified Eagle's medium – DMEM-high glucose and F12 Nutrient Mixture (Ham; Invitrogen), 10 % fetal calf serum (FCS, Sigma-Aldrich), 5 µg/ml insulin (Sigma-Aldrich), 0.4 µg/ml hydrocortisone (Sigma-Aldrich), 8.4 ng/ml cholera toxin (Sigma-Aldrich), 24 µg/ml adenine (Sigma-Aldrich) and 10 ng/ml epidermal growth factor (EGF, Invitrogen). H295R cells obtained from ATCC served as control and were cultured in DMEM:F12 medium supplemented with 1x insulin-transferrin-selenium (ITS) and Nu-serum (2.5 %). Mycoplasma contamination was excluded at regular intervals by using the Venor®GeM Classic Mycoplasma Detection Kit (Minerva Biolabs).

Cell experiments

2×10^7 JIL-2266 (passage (p) 25) and H295R cells (p 23) were harvested and washed twice with Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich) at 1000 rpm for 5 min at room temperature (RT). For induction of oxidative damage, H295R cells were treated with 100 µM hydrogen peroxide (H_2O_2) for 20 min at 4°C and subsequently washed twice with DPBS (1000 rpm, 5 min, RT). After the last washing step, cell pellets were resuspended in 2 ml paraformaldehyde (4 %) and kept in gentle movement at 4°C overnight. Suspensions were pelleted at maximal speed (15.000 rpm) using a microcentrifuge for 5 min. Pellets were incubated in 2 ml 70 % ethanol (EtOH) for 20 min and centrifuged at 1000 rpm for 5 min. This was repeated with 85 %, 96 % EtOH and 100 % isopropanol. After removal of the supernatant, 1 ml paraffin was added to cell pellets and the sample placed into a paraffin tank for 1h at 60°C. Subsequently, embedded pellets were stored until slides were prepared.

For mitotane treatment, 3×10^4 JIL-2266 cells were seeded in black 96-well plates with clear bottom. After 24 hours, cells were treated for another 24 h with mitotane (AlsaChim) dissolved in EtOH and viability was determined with CellTiter Glo Assay (Promega) as described before [36].

Chromogenic immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) slides of tumour tissue and cell pellets were deparaffinised twice in xylol for 25 min and subsequently rehydrated. Antigen retrieval was performed in 10 mM citric acid monohydrate buffer (pH 6.0) for 13 min under pressure and endogenous peroxidase blocked with 3 % H₂O₂. Unspecific binding sites were blocked with 20 % human AB serum for 1 h at RT. Primary antibodies against SF-1 (R&D Systems, N1665, 1:200), Ki-67 (Dako, MIB-1, 1:800), Inhibin α (Serotec, R1, 1:20), PD-L1 (Cell Marque, 28-8, 1:100) and TP53 (Dako, DO-7, 1:200) were incubated for 1 h at RT. Signal amplification was achieved by the Advance HRP Link Kit for 40 min and slides developed with the DAB+ Liquid Kit (Dako) for 10 min. Nuclei were counterstained using Mayer's haematoxylin for 10 min and blued for 5 min in running tap water.

Immunofluorescence

FFPE slides were deparaffinised three times in xylol for 5 min and subsequently rehydrated. Slides were incubated with proteinase K for 30 min at RT and afterwards with 100 μ g/ml RNase A for 1 h at 37 °C. DNA was denatured in 2 N HCl for 5 min and neutralized with 1 M Tris-base. Unspecific binding sites were blocked in 10 % goat serum for 1 h. Incubation with primary antibody (8-oxoG, Trevigen, #15A3), occurred in a dilution of 1:250 in DPBS with 0.1 % BSA (w/v) at 4 °C overnight. The primary antibody was omitted in negative controls. Secondary antibody (goat-anti-mouse Alexa Fluor488, ThermoFisher) was incubated in the dark for 1h at RT at a dilution of 1:200 in PBS with 0.1 % BSA (w/v). Nuclei were stained with DAPI (1:1000) for 3 min in the dark, slides washed with DPBS and mounted with ProLong Gold Antifade (ThermoFisher). Analyses of immune cell infiltrates were performed as described previously [31].

Microscopy

Microphotographs were taken with the Leica Aperio slide scanner (20 x objective) and processed with Aperio Image Analysis software (chromogenic immunohistochemistry) or the Zeiss Axioscope.A1 microscope (40 x objective) equipped with a Zeiss AxioCam 503 mono (immunofluorescence).

Extraction of DNA and RNA, qPCR

Pathological anatomical assessment confirmed high tissue quality and the presence of 90 % vital tumour cells by haematoxylin-eosin staining. Genomic DNA was isolated from fresh frozen ACC tumour, cell line (p 6, 8, 13, 29) and patient-matched leukocytes by using the Maxwell RSC Blood DNA Kit (Promega). DNA concentration was determined by Nanodrop spectrophotometer (Thermo Fisher).

RNA from fresh frozen tissue and cell lines was isolated for gene expression analyses with the Maxwell RSC Simply RNA Kit (Promega). RNA concentration was determined by Nanodrop Spectrophotometer (Thermo Fisher) and 1000 ng RNA were reverse transcribed with the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative reverse transcriptase PCR (RT-qPCR) was performed using TaqMan gene expression probes (Thermo Fisher Scientific) for *MC2R* (Hs00300820), *PINK1* (Hs00260868), *BUB1B* (Hs01084828), *CTNNB1* (Hs00355049), *SREBP1* (Hs01088691), *SREBP2* (Hs01081784), *CYP11B1* (Hs01596404), *CYP11B2* (Hs01597732), *STAR* (Hs00264912). Endogenously expressed *ACTB*

(Hs99999903) was used as housekeeping gene for normalization. For each RT-qPCR reaction, 5 ng cDNA were used and each sample was analysed in technical duplicates. All transcripts were amplified using TaqMan Gene Expression Master Mix (Thermo Fisher) using the CFX96 real-time thermocycler (Bio-rad) and the Bio-rad CFX Manager 2.0 software. Cycling conditions were 95 °C for 3 min, followed by 39 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 30 sec. Fold change was calculated using the Δ CT method, normalized to housekeeping gene *β -actin*.

Short tandem repeat (STR) profiling

DNA from JIL-2266 cells and corresponding leukocytes from donor patient was extracted using the Maxwell RSC Blood DNA Kit (Promega). Typing was performed using the STR-loci VWA, TH01, TPOX, CSF1PO, D16S539, D13S317, D7S820, D5S818 and the amelogenin sex-determining marker (AMEL-locus) to show patient originality, analyse congruousness and to rule out cross-contamination in the course of cultivation after p 5, 8, 13, and 29. Loci were amplified and electrophoretic analysis was carried out with the GeXP-instrument (AB Sciex). Data were analysed using the GeXP fragment analysis software.

Proliferation assay

1×10^5 JIL-2266 (p 30, 32, 33) and NCI-H295R per well were seeded into a 12-well plate. After 24 h, 48 h and 72 h, cells were harvested with trypsin and numbers were quantified in duplicates by using the Countess® Automated Cell Counter (ThermoFisher). Doubling time was determined using the exponential growth function in GraphPad Prism 8 software [40].

LC-MS/MS measurement of steroid hormones

JIL-2266 cells (2×10^6) were plated in 6-well plates in 2 ml DMEM/F12 medium supplemented with 5 % FCS and P/S for 48 h and stimulated with 10 μ M forskolin (Santa Cruz Biotechnology) or 10 ng/ml EGF (Invitrogen), 5 μ g/ml insulin (Sigma-Aldrich) and 24 μ g/ml adenine (Sigma-Aldrich) for 48 h. Supernatants were collected and steroids were quantified with the MassChrom steroids kit (Chromsystems) on a Qtrap 6500+ (Sciex) mass spectrometer coupled to a 1290 Infinity HPLC System (Agilent). Signal analysis was performed with Analyst Software (1.6.3, Sciex) as described elsewhere [41].

Library preparation

SureSelectXT Human All Exon V6 Kit (Agilent) was used for library preparation. Paired end sequencing with a read length of 100 base pairs (bps) was performed on a NovaSeq 6000 (Illumina). For the library preparation of the tumour and matched control sample, the xGen Exome Research Panel v2 (Integrated DNA Technologies) was used and paired end sequencing with a read length of 150 bps was performed on a NextSeq 500 (Illumina).

Bioinformatics

After an initial quality assessment using FastQC, v0.11.5 (Andrews S., 2010. available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>), adapters and low-quality reads were trimmed with TrimGalore, v0.4.0 (Krueger, F., 2012: available online at: http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) powered by Cutadapt, v1.8 [42]. The trimmed reads were mapped to the UCSC human genome (hg19) with BWA mem, v 0.7.17 [43], sorted and indexed using Picard, v1.125 (available online at: <http://broadinstitute.github.io/picard/>) and SAMtools, v1.3 [44] respectively. Duplicates were marked with Picard. For coverage calculations and base quality score recalibration, GATK3, v3.5 and GATK4, v4.0.11.0 [45] were used, respectively.

For germline variant calling, we used GATK3 and GATK4. MuTect2 integrated in the GATK4 package was used for somatic variant calling. All variants were annotated with ANNOVAR, v2019-10-24 [46] and considered if they were below a frequency of 2 % in the databases 1000g2015aug_all, ExAC_nontcga_ALL, gnomAD_exome_ALL and gnomAD_genome_ALL, if the position is covered by at least 20 reads and the alternative allele is covered by at least 8 reads and comprised at least 10 % of the total reads. The filtered MuTect2 results were used for TMB calculations. Mutational signatures were identified using MutaGene [47]. For this analysis, synonymous variants were also included.

Results

Case presentation

The donor of the tissue was a female 50 years old patient who presented with severe Cushing's syndrome including hypertension, muscle weakness, hypokalaemia and lymphopenia. Computer tomography scan indicated a left adrenal mass with a maximum of 9 cm diameter and pulmonary metastases. Endocrine work-up according to current guidelines [4] revealed androgen and cortisol excess (including pathological dexamethasone suppression test). Adrenolytic therapy with mitotane and metyrapone was initiated to ameliorate Cushing's syndrome, followed by left adrenalectomy two weeks later. Routine histological examination confirmed the diagnosis of an advanced high-grade ACC (35 mitoses / 10 high power fields (HPF)), Ki67 proliferation index of 60% up to 90% and a Weiss score of 9. By immunohistochemistry, the tumour was positive for steroidogenic factor-1 (SF-1) and partially for inhibin α . In line with our previous study of immune cells in ACC [31], the donor tumour was infiltrated by CD3⁺, CD4⁺ and CD8⁺ T lymphocytes to a moderate extent (7.8, 3.3 and 5.2 T cells per HPF, respectively; **Figure 1 A**). Programmed cell death-ligand 1 was expressed in less than 1% of tumour cells and DNA MMR protein expression was normal. After surgery, the patient received chemotherapy with EDP in addition to mitotane. After two cycles, the patient experienced rapid progression of pulmonary metastases, new liver metastases and died shortly thereafter.

The family history was remarkable with a cerebellar tumour not otherwise specified in the mother and breast cancer in the sister and grandmother. LFS was excluded by routine germline sequencing of TP53.

Establishment of the JIL-2266 ACC cell line

Tumour material was obtained from surgical resection and subsequently enzymatically processed. By using chemically modified polystyrene Primaria culture flasks with a 1:1 ratio of nitrogen and oxygen and without CO₂ supply, the generated cell suspension was initially cultured in the presence of ROCK inhibitor supporting immortalization of human tumours [39]. Subsequently, cells initially grew in primary culture preferably in colonies. By means of weakly renewal of media up to one half, cells started to proliferate and were passaged first after 180 days. While the time interval for passaging decreased to 90 days, fibroblast removal was performed after further 270 days at p 4. By consistent appearance, the new cell line proliferated until confluence for further 50 days. At that point, the cells were termed JIL-2266 and routinely passaged with further declining intervals (from 168 h to 41 h) and characterised.

To confirm match of the cell line and the corresponding human blood sample, STR profiling was performed. JIL-2266 cells only exhibited alleles expressed in the corresponding human blood DNA, confirming the authenticity and correspondence between cell line and respective human tissue. For three loci - THO1 (chromosomal localization: 11p15.5; intron 1 tyrosine-hydroxylase gene), D16S539 (chromosomal localization: 16q24.1) and TPOX (chromosomal localization: 2p25.3; intron 10 thyroid-peroxidase gene) – the JIL-2266 cell line demonstrated loss of heterozygosity (LOH) (**Table 1**). Comparison with large cell line STR profile databases (DSMZ online STR analysis <https://www.dsmz.de/services/human-and-animal-cell-lines/online-str-analysis>) provided the proof of a unique genomic identity and excluded cross-contamination.

Immunohistochemistry reveals similarity between primary tumour and JIL-2266 cells

For confirmation of the adrenocortical origin of JIL-2266 cells and comparison with the standard ACC cell line H295R, immunohistochemistry of the primary tumour and cell pellets was performed. SF-1 was moderately expressed in the corresponding primary tumour and in JIL-2266 cells, while H295R cells showed a very strong SF-1 expression (**Figure 1 B**). The primary tumour showed only partial expression of inhibin α with very localized positive areas, while inhibin α expression was completely absent in the corresponding cell line JIL-2266. H295R cells showed strong inhibin α expression (**Figure 1 C**). Ki-67 and TP53 expression were high in the primary tumour, JIL-2266 and H295R cells (**Figure 1 D and E**). Analysis of cell pellets revealed a larger size of JIL-2266 compared to H295R cells (**Figure 1**). JIL-2266 cells grow adherent to the plastic bottom of the culture flask and morphological investigation of p 8, 14 and 30 confirmed morphological stability during multiple passaging steps and over time (**Figure S1**).

mRNA expression of adrenal gland markers

Expression of adrenocortical markers transcripts by qPCR showed similar expression of *PINK1*, *BUB1B* and *CTNNB1* in the matching primary tumour and JIL-2266 cells with insignificant differences between passages (**Figure 2**). *BUB1B* was overexpressed in all ACC samples compared to normal adrenal gland (nAG). Expression of adrenal cortex markers was variable and lower in all ACC samples compared to nAG. *MC2R* expression was undetectable in JIL-2266 cells and highest expression of steroidogenic markers were observed for *SREBP1* and *SREBP2* (**Figure 2**).

Sensitivity to mitotane

The proliferation rate of the H295R was 36 ± 15 hours (**Figure 3 A**) and 41 ± 13.6 hours for the JIL-2266 (p 30, **Figure 3 B**). The EC_{50} value for 24 h mitotane treatment was significantly higher with 8.3×10^{-5} M (**Figure 3 C**) in JIL-2266 compared to H295R cells (1.8×10^{-5}) [48].

Steroid hormone profiling

LC-MS/MS analysis of steroid hormones in standard cell culture medium showed undetectable quantities of adrenal steroid hormones independent of the passage (**Table S1**). 48 h treatment with forskolin did not result in detectable steroidogenesis of JIL-2266 cells (**Table S1**). Yet, when JIL-2266 cells were cultured with adenine, insulin, EGF and cholera toxin, small amounts of the androgens and the precursors progesterone, 17-OH-progesterone, DHEA, androstenedione and testosterone were detectable, which decreased with higher passages (**Figure S2** and **Table S1**). These *in vitro* data are consistent with the plasma steroid hormone profile in the donor who had increased androstenedione, testosterone and 17-OH-progesterone.

Whole exome sequencing

Exome sequencing revealed the presence of a pathogenic germline mutation in the *MUTYH* gene (*MUTYH*: NM_012222.2:exon3:c.316C>T:p.R106W), encoding the mutY DNA glycosylase.

By comparing exome sequencing data from primary tumour and the JIL-2266 cell line with donor leukocytes DNA as reference, 556 somatic genetic variants - single nucleotide variants and small insertions/deletions (InDels) – were found in the primary tumour and 683 variants in JIL-2266 cells, with 512 shared alterations. The number of genetic variants increased from 556 SNVs/InDels per exome in the primary tumour to 683 in JIL-2266 cell line (**Figure 4 A**). *TP53* was hemizygous for a stop gain mutation (*TP53*: NM_000546.5:exon8:c.859G>T:p.E287X) both in the patient's primary tumour and JIL-2266 cell line. Additionally, a LOH in the *MUTYH* gene (*MUTYH*: NM_012222.2:exon3:c.316C>T:p.R106W) in both primary tumour and cell line compared to patient blood DNA was found (**Figure 4 B**). While both the primary tumour and JIL-2266 cell line held a wild type *CTNNB1* allele, they showed a somatic nucleotide variants in the *ZNRF3* gene (*ZNRF3*: NM_001206998.2:exon8:c.2569G>T:p.G857W). The complete list of somatic alterations and their characteristics is shown in supplementary **Table S2**. By applying gene signature analysis, we found prevalent cytosine (C) > adenine (A) single base substitutions corresponding to signature 18 in the Catalogue of Somatic Mutations in Cancer (COSMIC) database (https://cancer.sanger.ac.uk/cosmic/signatures_v2.tt) in line with *MUTYH* deficiency.

Functional assessment of the pathogenic MutY DNA glycosylase

One of the most frequent types of oxidative DNA damage is 8-oxoguanine (8-oxoG) formation. 8-oxoG mispairs with adenine during replication [49], which is counteracted by base excision repair (BER) in a complex that includes MutY. Mutations that compromise MutY function hence lead to the accumulation

of 8-oxoG leading to an increase of G:C>T:A transversions. We performed immunocytochemistry of 8-oxoG in FFPE tissues of the corresponding primary tumour and a *MUTYH* WT ACC and in parallel, in cell pellets of JIL-2266, H295R and H295R cells pre-treated with H₂O₂ to induce oxidative damage. H295R cells without prior H₂O₂ treatment were negative for 8-oxoG (**Figure 5 A**), while H₂O₂ treatment led to a marked accumulation of 8-oxoG in NCI-H295R cells (**Figure 5 B**). Untreated JIL-2266 cells (**Figure 5 C**) and fresh frozen tumour (**Figure 5 D**) stained strongly positive for 8-oxoG while ACC with *MUTYH* WT allele was completely 8-oxoG negative, in line with our expectation of a causative role of *MUTYH* mutation for high TMB with COSMIC signature 18 (**Figure 5 E**).

Taken together, this novel human ACC cell line exhibits high TMB caused by oxidative DNA damage that fails to be counteracted by BER due to pathogenic *MUTYH* mutation.

Discussion

ACC is a very rare and heterogeneous malignancy and the development of new and effective treatment options is significantly hampered by the lack of pre-clinical models that mirror the clinically heterogeneous picture of the disease. Here we describe a novel patient-derived ACC cell line, JIL-2266, which is to our knowledge the first cell line since 30 years that was established by direct transfer into cell culture. Interestingly, JIL-2266 harbour a high TMB due to a pathogenic *MUTYH* mutation.

SF-1 positivity is an essential marker of adrenal cortical origin [50]. Both JIL-2266 cells and its corresponding tumour show moderate SF-1 expression (Figure 1 B). JIL-2266 cells also express additional adrenal markers involved in steroidogenesis albeit at lower levels than H295R cells; however, both less in comparison to normal adrenal glands. This is in line with low expression of steroidogenic enzymes and absent steroid hormone secretion in the JIL-2266 cell line under basal conditions; only seemingly in contrast to the clinical presentation of the patient who had pre-operative pathological elevation of steroid blood concentration. Indeed, the very high tumour burden in the presence of rather low steroidogenic capacity of tumour cells may explain the discrepancy. In general, steroid hormone secretion is variable in the JIL-2266 cells and similarly dependent on cell culture supplements and passage as in H295R cells [41]. Lower steroid secretion was associated with higher passages (Figure S2). It is conceivable that the accumulation of mutations with increasing passage negatively affects the cells' steroidogenic capacity. ACC markers *PINK1*, *BUB1B* and *CTNNB1* were lower in the JIL-2266 cell line compared to H295R cells. Yet, expression was similar to that in the primary tumour.

Within the last few years, three new ACC cell lines [34, 35] were developed in addition to the *bona fide* cell line H295R, which has been excessively studied since its establishment in the early 1990s [32]. All available cell lines have mutations in *TP53* (MUC-1, CU-ACC2), *CTNNB1* (CU-ACC1) or both (H295R) [33, 34, 35]. JIL-2266 cells are not an exception as they also harbour a probably hemizygous *TP53* mutation (NM_000546.5:exon8:c.859G>T: p.E287X).

Mutations in DNA repair mechanisms have been found in a small proportion of ACC and mostly affect DNA MMR genes. *MUTYH* encodes a MutY DNA glycosylase and is part of the BER involved in the recognition and resolution of 8-oxoG-adenine mismatches by excising the mis-paired adenine. Sporadic cases of ACC have been shown to harbour *MUTYH* mutations. Pilati et al. described two hyper-mutated ACC tumours dominated by C>A transversions and germline *MUTYH* mutation (ACC33: NM_001128425.1:c.1187G>A, p.Gly396Asp and ACC39: NM_001128425.1:c.721C>T, p.Arg241Trp) [13]. Additionally, by analysing the TCGA cohort consisting of 91 ACCs, once again two tumours with COSMIC signature 18 and rare pathogenic *MUTYH* mutations (A: NM_001128425.1:c.467G>A; p.Trp156* and B: NM_001128425.1:c.536A>G, p.Tyr179Cys) were observed [11, 30].

Yet, the *MUTYH* (NM_012222.2:exon3:c.316C>T: p.R106W) mutation identified in our ACC patient and the derived JIL-2266 cell line has not been described in ACC but was observed in colorectal cancer [51]. This mutation was heterozygous in the germline of the patient and became hemizygous in the tumour. The observed strong accumulation of 8-oxoG in the primary tumour and JIL-2266 cell line, to a comparable extent as observed in H295R cells exposed to oxidative stress (H₂O₂ treatment), suggests a strong impairment in MutY DNA glycosylase function due to the mutation identified. Impaired DNA repair is further indicated by the increase of TMB in the JIL-2266 cell line after excessive passaging and replications compared to the primary tumour as revealed by exome sequencing. Our cell line exhibited the distinctive COSMIC mutational signature 18 associated with oxidative DNA damage and characterised by an enrichment of C>A transversions [30, 49] caused by *MUTYH* mutations. COSMIC signature 18 has been rarely described in ACC and all these tumours were found to harbour *MUTYH* mutations [30].

PD-1 checkpoint inhibition may be particularly valuable in patients with DNA repair defects; the PD-1 inhibitor pembrolizumab is FDA-approved for MMR deficient or microsatellite instability high (MSI-H) solid tumours regardless of its origin. ACC has been shown to be associated with germline mutations in DNA MMR genes and hence LS-associated tumour [21, 52]. While the overall response to immunotherapy in ACC patients is heterogeneous [24, 27] and not certainly associated with PD-L1 expression status [27], results from clinical trials [27] and case reports of successful PD-1 inhibition in MMR deficient patients are encouraging [53]. Hence, our novel cell line, derived from a primary tumour with present tumour-infiltrating lymphocytes also shows a deficient DNA repair mechanism and high TMB, which combined present a unique model to study antitumoural response to immunotherapy *in vitro*. Accordingly, a MMR deficient, high TMB humanized CU-ACC2 PDX demonstrated a significant increased immune infiltration following PD-1 inhibition and tumoural response to treatment [28].

Consistent with failure of the patient's tumour to respond to mitotane treatment, JIL-2266 cells were unresponsive to mitotane in culture. The patient's rapid disease progression precluded a therapeutic attempt with immune checkpoint inhibitors that have been demonstrated to be effective in other *MUTYH* mutated tumours [29].

Conclusion

The newly developed JIL-2266 ACC cell line presents an additional and valuable tool reflecting the genetic heterogeneity of ACC. JIL-2266 will enable a better understanding of mutY DNA glycosylase function in ACC and allows to study further the mechanisms of resistance to mitotane. Moreover, this new ACC cell line exhibits high TMB and COSMIC signature 18 and will be valuable for the assessment of mechanisms underlying response and resistance to immunotherapy in ACC.

MUTYH should be included in the genetic counselling and testing strategy of ACC to identify patients who potentially respond to immunotherapy [29, 52, 53, 56].

Abbreviations

°C	degree Celsius
17-OH progesterone	17 α -hydroxyprogesterone
8-oxoG	8-oxoguanine
A	adenine
ACC	adrenocortical carcinoma
ACTB	beta-actin
BER	base excision repair
BPS	base pairs
BSA	bovine serum albumin
C	cytosine
CD3	cluster of differentiation 3
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
cDNA	complementary Deoxyribonucleic acid
CO ₂	carbon dioxide
COSMIC	catalogue of somatic mutations in cancer
CTNNB1	β -catenin 1
CYP11B1	cytochrome P450 11B1
CYP11B2	cytochrome P450 11B2
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DHEA	dehydroepiandrosterone
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EC ₅₀	half maximal effective concentration
EDP	etoposide, doxorubicin and cisplatin
EGF	epidermal growth factor
ENSAT	European Network for the Study of Adrenal Tumours

EtOH	ethanol
FCS	fetal calf serum
FDA	food and drug administration
FFPE	formalin-fixed-paraffin-embedded
h	hour
H ₂ O ₂	hydrogen peroxide
HCl	hydrochloric acid
HPF	high power fields
HRP	horseradish peroxidase
IGF2	insulin like growth factor 2
IGF-R1	insulin like growth factor receptor 1
InDels	Insertion/deletions
ITS	insulin-transferrin-selenium
LC-MS/MS	liquid chromatography-mass spectrometry - tandem mass spectrometry
LFS	Li-Fraumeni syndrome
LOH	loss of heterozygosity
LS	Lynch syndrome
MC2R	melanocortin receptor 2
min	minute
MLH1	mutL homolog 1
MMR	mismatch repair
MSH2	mutS homolog 2
MSH6	mutS homolog 6
MSI	microsatellite instability
MSI-h	microsatellite instability high
MUT	mutant
MUTYH	mutY DNA glycosylase
nAG	normal adrenal gland
p	passage

P/S	penicillin G/streptomycin
PD-1	programmed cell death 1
PD-L1	programmed cell death ligand 1
PDX	patient-derived xenograft
PINK1	PTEN-induced kinase 1
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RNAse A	ribonuclease A
ROCK	rho-associated protein kinase
rpm	rounds per minute
RT	room temperature
RT-qPCR	real-time quantitative polymerase chain reaction
SF-1	steroidogenic factor-1
SNV	single nucleotide variant
SREBP1	sterol regulatory element-binding transcription factor 1
SREBP2	sterol regulatory element-binding transcription factor 2
STAR	steroidogenic acute regulatory protein
STR	short tandem repeat
TMB	tumour mutational burden
TP53	tumour suppressor p53
WT	wild type
ZNRF3	zinc and ring finger 3

Declarations

Ethics approval and consent to participate

All patients included were participants of the ENSAT (European Network for the Study of Adrenal Tumours) registry and biobank. The study conformed to the principles of the Declaration of Helsinki, the Good Clinical Practice Guidelines and was approved by the Ethics Committee of the University of Würzburg (# 88/11). All patients provided written informed consent for the use of tissue, cells, clinical data and genetic characterisation.

Consent for publication

This manuscript does not contain personal and/or medical information about identifiable living individual. All patients were anonymised and provided written informed consent.

Availability of data and materials

All data generated or analysed during relevant to the current study are included in the article, uploaded as supplementary information or are available from corresponding author on reasonable request.

Competing Interests

The authors declare no competing interest.

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Author's contributions

IW and MK participated in the study design and supervision. MF and MK were responsible for funding acquisition. LSL, JS, SA, SH, SK and IW performed the experiments and are involved in data analyses. LSL, JS, MK and IW prepared and edited the final manuscript. All authors read and approved the final manuscript for publish.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Immunofluorescence staining (40 x magnification) and quantification of CD3⁺, CD4⁺ and CD8⁺ tumour-infiltrating lymphocytes of patient-derived primary ACC (coloured dots) compared to other primary ACCs (A). Immunohistochemistry of FFPE tissue of the primary ACC and cell pellets from JIL-2266 and H295R cells and morphological investigation of JIL-2266 cells. (B) SF-1 staining, (C) inhibin α staining, (D) Ki-67 and (E) TP53 staining, scale bars: 100 μ M.

Figure 2

mRNA expression of different ACC markers (BUB1B, PINK1 and CTNNB1) and steroidogenic markers in the JIL-2266 cell line, its corresponding primary tumour, H295R cells and five normal adrenal glands (nAG), analysed by RT-qPCR.

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Figure 3

Proliferation of JIL-2266 cells and their response to mitotane treatment. (A) JIL-2266 cells proliferate in culture, yet at a lower rate than H295R cells. Doubling time of JIL-2266 cells is 41 h while H295R cells have a doubling time of 36 h. (C) JIL-2266 were unresponsive to mitotane treatment with an EC50 value of 8.3×10^{-5} after 24 h.

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Figure 4

Whole exome sequencing of human primary tumour, JIL-2266 cell line and leukocyte reference. (A) Tumour mutational burden (TMB) with 10 % variant allele frequency cut-off increases from primary tumour to JIL-2266 cell line, while mutational signature remained stable. (B) Exome sequencing analysis of human tumour, JIL-2266 cell line and blood demonstrated evidence of TP53 and MUTYH mutation in primary tumour and corresponding JIL-2266 cell line.

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Figure 5

Immunofluorescence staining of 8-oxoguanine in FFPE cell pellets and ACC tissues. (A) Untreated H295R cells stained negative for 8-oxoguanine, while (B) H₂O₂ treatment strongly increased 8-oxoguanine staining positivity. (C) Untreated JIL-2266 cells showed a strong 8-oxoguanine staining positivity as the corresponding primary tumour (D). (E) In a MUTYH WT ACC, 8-oxoguanine staining was negative. DAPI was used to stain nuclei.

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