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Adrenocortical cancer cell line mutational profile reveals aggressive genetic background

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Abstract
Adrenocortical carcinomas are rare tumors with poor prognosis and limited treatment options. Although widely used as in vitro models to test novel therapeutic strategies, the adrenocortical carcinoma-derived cell lines NCI-H295R and SW-13 have only partially been described genetically. Our aim was to characterize the mutational landscape of these cells to improve their experimental utility and map them to clinical subtypes of adrenocortical carcinoma. Genomic DNA from NCI-H295R and SW-13 cells was subjected to whole-exome sequencing. Variants were filtered for non-synonymous mutations and curated for validated adrenocortical and pan-cancer driver gene mutations. Genes mutated in the cell lines were mapped using gene ontology and protein pathway tools to determine signaling effects and compared to mutational and clinical characteristics of 92 adrenocortical carcinoma cases from The Cancer Genome Atlas. NCI-H295R and SW-13 cells carried 1325 and 1836 non-synonymous variants, respectively. Of these, 61 and 76 were known cancer driver genes, of which 32 were shared between cell lines. Variant interaction analyses demonstrated dominant TP53 dysregulation in both cell lines complemented by distinct WNT (NCI-H295R) and chromatin remodeling (SW-13) pathway perturbations. Both cell lines genetically resemble more aggressive adrenocortical carcinomas with worse prognosis, for which development of targeted therapies is most critical. Careful incorporation of the genetic landscapes outlined in this study will further the in vitro utility of these cell lines in testing for novel therapeutic approaches for adrenocortical malignancy.

Introduction
Adrenocortical cancer (ACC) is a rare endocrine tumor with a poor prognosis. Recent estimates put the incidence at around one case per million people per year, with overall 5-year survival around 40% (Nicolson et al. 2018). Current non-surgical treatment options include radiotherapy, cytotoxic chemotherapy and the adrenolytic mitotane, but no targeted agents are currently widely available for treatment of these tumors (Varghese & Habra 2017). Margin-negative resection remains the only approach for a durable cure in most cases. Although some reports highlight modest improvements in survival for early stage disease in the last decade, there is still significant opportunity for improvement through the development of targeted molecular therapies, which have been effective in a number of other solid tumors (Fassnacht et al. 2010, Konda & Kirschner 2016).
Recent genomic analyses supported the previously implicated roles of dysregulated WNT and TP53 pathways in these tumors, representing opportunities for development of targeted therapies, though none have reached routine clinical practice to date (Assie et al. 2014, Juhlin et al. 2015, Konda & Kirschner 2016, Zheng et al. 2016). Chromatin remodeling abnormalities, some previously not described in the pre-genomics era, were also noted in adrenocortical cancers in these studies. ACC is noted to carry a moderate number of total point mutations relative to a pan-cancer data set and relatively high mutational burden relative to other endocrine malignancies (Chalmers et al. 2017). Additionally, copy number changes are frequent in ACC, often constituting an alternative genetic pathway affecting the same tumor suppressors (for copy losses) and oncogenes (for copy gains) as the typical single nucleotide variants (Assie et al. 2014, Juhlin et al. 2015, Zheng et al. 2016).

Investigators have chiefly relied on two widely used ACC cell lines, NCI-H295R and SW-13, for in vitro studies of the mechanisms of adrenocortical carcinogenesis and to test the efficacy of novel therapeutic agents (Brown et al. 2016, 2018, Murtha et al. 2016, Cheng et al. 2017). NCI-H295 cells were originally derived from a female patient who presented with a large ACC, which was later metastatic to liver and lung, and the NCI-H295R cell line was developed subsequently to allow for growth in monolayer tissue culture; SW-13 cells were derived from a carcinoma of small cell type in the adrenal cortex of a middle-aged woman and has been subject to some controversy as to the cell of origin given the unusual histology (Table 1) (Rainey et al. 2004, Wang & Rainey 2012). Both cell lines have been previously shown to harbor loss-of-function TP53 alterations (K193Y single nucleotide variant in SW-13 and large homozygous deletion of exon 8 and 9 in NCI-H295R); furthermore, unlike non-hormone-producing SW-13 cells, NCI-H295R cells can be induced to produce steroid hormones and are known to harbor a gain-of-function CTNNB1 mutation (Tissier et al. 2005, Cerquetti et al. 2008, Ismail & Bateman 2009, Sampaoli et al. 2012, Wang & Rainey 2012). Beyond the previous selected structure/function studies on a limited number of candidate genes, little is known about the comprehensive genetic background of these commonly used ACC cell lines. The underlying genetic and signaling abnormalities inherent to these cell lines are therefore largely overlooked in most of these studies.

Moreover, the lack of comprehensive knowledge about the genomic landscape of these commonly used cell lines potentially limits their utility in developing effective molecular therapies for ACC treatment. Further, investigators aiming to target a particular pathway with a novel agent will be faced with confusing experimental results due to unforeseen and unknown genetic underpinnings of the model cell lines. Because cell lines can accumulate additional genetic and epigenetic changes in culture over time, a cell line may in theory deviate significantly from the original cancer from which it was derived, though previous studies have shown this effect to be relatively minor at least in terms of driving mutations (Qiu et al. 2016). In vitro results, thus, are at risk of having limited clinical applicability. Particularly in an environment of growing interest in precision medicine and personalized oncology, developing tailored therapeutic modalities against particular molecular subtypes of ACC shows promise (Jouinot & Bertherat 2018). The aim of the present study was to characterize the mutational profile of these widely used ACC cell lines via whole-exome sequencing analysis, to both confirm their validity as model systems of ACC and help researchers in the interpretation of in vitro molecular studies of novel targeted therapeutics.

### Materials and methods

Authenticated (STRS-verified) NCI-H295R and SW-13 cells were purchased from the American Type Cell Collection and grown under standard sterile culture conditions at 37°C and 5% ambient CO₂ in a standard humidified incubator (Brown et al. 2018). Culture medium for SW-13 consisted of Dulbecco’s modified Eagle’s medium (DMEM) with 10% v/v fetal bovine serum and 10,000 units/mL

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Cell line characteristics.</th>
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<tbody>
<tr>
<td>Cell line</td>
<td>Patient</td>
</tr>
<tr>
<td>SW-13</td>
<td>55, F</td>
</tr>
<tr>
<td>NCI-H295R</td>
<td>48, F</td>
</tr>
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</table>

ACC, adrenocortical carcinoma; DMEM, Dulbecco’s modified Eagle medium; F, female.
penicillin/streptomycin, while medium for NCI-H295R was DMEM/F12 with 5% v/v NuSerum, 0.1% v/v insulin–transferrin–selenium and 10,000 units/mL penicillin/streptomycin. Genomic DNA was harvested from early passage cells (4–5 passages) with the DNeasy Blood and Tissue DNA kit (Qiagen) according to manufacturer’s instructions. No institutional review board approval was required or obtained, since no live human or animal subjects were used in the study.

The genomic DNA was then subjected to whole-exome sequencing via an established exome library preparation protocol and the Illumina sequencing platform, with sequence alignment and single nucleotide variant and insertion–deletion calling performed as previously described, using the same methods employed in a recent study of patient ACC exomes from our group (Juhlin et al. 2015). As there is no matched normal tissue available for these cultured cells, the human reference genome was used to generate sequence variant calls, as previously described in other cell line sequencing studies (Vandamme et al. 2015). Known common variants in annotated databases 1000 Genomes and the National Heart, Lung, and Blood Institute’s Exome Variant Server (NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) were excluded and the remaining sequence variants were mapped to the best protein sequence isoform available (1000 Genomes Project Consortium et al. 2015). Variants were annotated for damaging, non-synonymous single nucleotide variants and insertion/deletion events, using PhyloP, SIFT and PolyPhen-2 scores (Kumar et al. 2009, Adzhubei et al. 2010, Pollard et al. 2010). Predicted mutations were matched with known patient-derived ACC driver genes as identified in the three most recent next-generation sequencing studies in ACC tumors, as well as validated cancer driver genes in other cancer types as curated by IntOGen and the COSMIC cancer gene census (Gonzalez-Perez et al. 2013, Assie et al. 2014, Juhlin et al. 2015, Zheng et al. 2016, Forbes et al. 2017). Genes identified as mutated in both cell lines that had not previously been described as cancer drivers were also noted.

Mutational profiles generated from ACC cell lines in this study were compared to previously published ACC exomes from The Cancer Genome Atlas (TCGA) to map each cell line studied to a subset of in vivo tumors (Zheng et al. 2016). Mutational and clinical parameters were downloaded from the UCSC Xena Browser (https://xenabrowser.net, accessed 8/1/2018). ACC tumors were assigned as ‘SW-13 type’ if they carried at least one non-synonymous variant in a gene found in both the cell line driver shared and SW-13 private mutation sets, and ‘NCI-H295R type’ if they met the same criteria in the shared and NCI-H295R private sets. These categories were compared to Weiss score, presence of metastases at presentation and overall survival to determine the expected clinical behavior of the ACCs modeled by the cell lines under investigation. The Mann–Whitney test was used for comparing Weiss scores, while Fisher’s exact test was used for proportions. Log-rank testing was used to compare survival figures. Statistical analyses were carried out using GraphPad Prism 7.

Cancer driver genes identified were subjected to gene ontology and protein pathway analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was used to identify signaling pathways affected by gene mutations in these cell lines (Kanehisa et al. 2017). STRING analysis uses networks of protein–protein interactions to find overlaps in large-scale genomics data sets (Szklarczyk et al. 2017). For the present study, KEGG and STRING analyses were performed on the sets of mutated driver genes carried by each cell line to determine which pathway perturbations were shared and which were unique. Similar analyses were performed for the total mutational profiles of each cell line, including suspected cancer driver and likely passenger mutations.

Results

Whole-exome analysis revealed 1325 and 1836 non-synonymous protein-coding or splice-site variants in NCI-H295R and SW-13, respectively, including both single nucleotide variants and short insertion/deletion events. Multiple sequence variants within the same gene in the same cell line were de-duplicated, leaving a total of 1028 and 1463 individual genes mutated in NCI-H295R and SW-13 genomes, respectively. Of these, 61 and 76 were known cancer driver genes in ACC or other cancer types, of which 32 were shared between the cell lines, and the remainder were private mutations (Supplementary Table 1, see section on supplementary data given at the end of this article).

Of the 23 potential ACC driver genes identified in patient exome studies, four were found to carry non-synonymous mutations in NCI-H295R and three in SW-13 (Fig. 1) (Assie et al. 2014, Juhlin et al. 2015, Zheng et al. 2016). No single nucleotide variant was identified in NCI-H295R in the TP53 tumor suppressor gene. However, NCI-H295R has been previously shown to carry a homozygous deletion of exons 8 and 9 of TP53, which is undetectable
by our exome sequencing pipeline; hence, we designated TP53 as ‘mutated’ in our study, for consistency with prior works and to reflect the biological reality in these cells (Cerquetti et al. 2008, Sampaoli et al. 2012). Both cell lines therefore carried CDC27 and TP53 mutations, while mutations in MLL4/KMT2D in SW-13 and ATRX and CTNNB1 in NCI-H295R were unique to each cell line.

Correlation of the cell line mutational profiles to the TCGA ACC genome data highlighted the well-characterized genetic heterogeneity of ACCs. In total, 12% (11/92) of TCGA ACC samples were classified as ‘SW-13-type’, while 16% (15/92) were classified as ‘NCI-H295R-type’. Six samples (6.5%) fell into both categories. Weiss score was significantly higher for SW-13 type tumors (median 7 vs 5, \( P < 0.04 \)) and for NCI-H295R-type tumors (median 7.5 vs 5, \( P < 0.01 \)) when compared to tumors not fitting into those categories (Table 2). SW-13-type tumors were more likely to be metastatic at presentation (50 vs 16\%, \( P < 0.03 \)). NCI-H295R-type tumors were also more frequently metastatic at presentation, but this trend was not statistically significant (36 vs 17\%, \( P = 0.14 \)). In terms of overall survival, SW-13 type tumors showed poor prognosis (\( P = 0.0005 \)), while NCI-H295R type tumors showed no difference in overall survival (\( P = 0.19 \)) (Fig. 2).

Gene interaction network analysis revealed potentially distinct signaling perturbations at work in each cell line, as well as some overlap. Mapping the predicted interactions between shared cancer driver genes revealed that both cell lines have in common dysregulation of TP53-regulated apoptosis pathways, similar to that reported in recent next-generation sequencing studies of ACC (Assie et al. 2014, Juhlin et al. 2015, Zheng et al. 2016). The private mutational network specific to SW-13 drivers centered around altered chromatin remodeling, specifically the SMARCA4 and EP300 genes and their networking partners, potentially deregulates the expression of multiple downstream target genes through histone modifications (Fig. 4) (NCBI Gene). NCI-H295R cells carried mutations in the WNT/β-catenin pathway, the classic signaling network repeatedly reported to be affected in ACC (Fig. 5) (Assie et al. 2014, Juhlin et al. 2015, Zheng et al. 2016).

Both cell lines’ driver mutations were enriched in KEGG ‘pathways in cancer’ and ‘PI3K/Akt signaling pathway’ genes but were not significantly enriched in other relevant pathways (Table 3). Pathway analysis of all non-synonymous variants, including both driver and passenger mutations, revealed enrichment of ECM–receptor interactions in NCI-H295R cells, while

Figure 1
ACC, pan-cancer and ACC cell line driver mutations. Driver mutations are noted in each cell line, compared to ACC driver mutations identified in the three largest whole-exome sequencing studies of ACC tumors. Of note, TERT mutations identified in prior studies have been in the promoter region, not sequenced for our study. Genes annotated in COSMIC or IntOGen are also noted. Blue color indicates mutated genes. *The NCI-H295R mutation in TP53 is a previously shown homozygous deletion of two exons, not detectable using whole-exome sequencing. A full colour version of this figure is available at https://doi.org/10.1530/JME-18-0262.
no additional KEGG pathway enrichment was noted for SW-13 cells. This likely reflects the limited selective advantage conferred by multiple hits within the same pathway, resulting in largely mutually exclusive mutational profiles for each specific network.

Table 2  Clinicopathological characteristics of TCGA cases corresponding to ACC cell lines.

<table>
<thead>
<tr>
<th></th>
<th>NCI-H295R type</th>
<th>Other</th>
<th>P</th>
<th>SW-13 type</th>
<th>Other</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median Weiss score (IQR)</td>
<td>7.5 (6–8.25)</td>
<td>5 (4–7)</td>
<td>0.0097</td>
<td>7 (5.5–8.5)</td>
<td>5/10, 50%</td>
<td>0.14</td>
</tr>
<tr>
<td>M1 disease</td>
<td>5/14, 36%</td>
<td>13/76, 17%</td>
<td></td>
<td></td>
<td>5 (3.5–7)</td>
<td>13/80, 16%</td>
</tr>
</tbody>
</table>

P values reflect two-tailed Mann–Whitney tests for Weiss scores, and Fisher’s exact tests for metastatic disease. IQR, interquartile range; M1, metastatic disease at presentation.

Discussion

This report of whole-exome sequencing of two widely used ACC cell lines revealed a mutational landscape largely overlapping with the genetic landscape of ACC tumors reported in recent next-generation sequencing studies and strongly supports the use of these cell lines in vitro as experimental models to explore the mechanisms driving the origin and progression of ACCs. Moreover, a clear understanding of the mutations enabling and/or disrupting multiple signaling networks at work in these cell lines will help investigators to design relevant experiments to test novel therapeutic strategies in the appropriate signaling contexts.

TP53 and CTNNB1 mutations are prevalent in ACC and were also found in these cell lines, along with many additional mutations (similar to the overall genetic chaos frequently observed in ACC). Other less common but still potentially important cancer driver mutations were also identified, with significant implications for interpretation of previous and future work utilizing these cell lines.

Figure 2
Survival analysis of SW-13 type and NCI-H295R type TCGA cases. (A) SW-13 type cases demonstrate markedly worse overall survival when compared to the remainder of the cohort (log-rank P = 0.0005). (B) NCI-H295R type cases demonstrate a trend toward worse survival, but this was not statistically significant (log-rank P = 0.19).

Figure 3
NCI-H295R and SW-13 shared driver mutations. Driver genes carrying non-synonymous mutations in both cell lines, with protein–protein connections, mapped using the STRING database (Szklarczyk et al. 2017). TP53 and related genes are the nexus in this network, common to both cell lines. Genes with no connections identified are not displayed. A full colour version of this figure is available at https://doi.org/10.1530/JME-18-0262.
the extensive genetic changes which can spontaneously occur in cell culture, we hoped to establish the similarity between these experimentally useful cell lines and the tumors for which they are a model system. Our study demonstrates that each cell line likely corresponds to a particular subset of ACCs which share perturbations along related pathways, and both of these genetic subsets exhibit more aggressive clinical behavior, particularly the SW-13-type tumors.

In particular, SW-13 may be a better model for ACC tumors with abnormalities in chromatin remodeling genes (such as KMT2D, SMARCA4 or EP300), while NCI-H295R may be a better model system for WNT/CTNNB1-altered tumors. Interestingly, some of these chromatin remodeling genes have been implicated in small-cell lung cancers, which could be considered to support the idea that this cell line may not truly be adrenal in origin, though these genes have been reported to be mutated in ACCs as well (Peifer et al. 2012, Zheng et al. 2016). Some subtypes of ACC, such as those with activating MAP kinase pathway mutations, may not be well-represented by these cell lines (Kotoula et al. 2009, Pereira et al. 2019). Functionally, NCI-H295R and SW-13 cells represent hormonally active and inactive ACCs respectively, and their distinct exomic profiles may reveal clues to their particular functional phenotypes. Notably, each cell line carried multiple mutations in dominant signaling networks that were shared with clinical ACCs, potentially indicating that they may behave similarly even in the absence of complete mutational overlap.

The overall mutational burden in these cell lines was quite high compared to ACC tumors reported previously (Assie et al. 2014, Juhlin et al. 2015, Zheng et al. 2016). Whether this discrepancy is due to the lack of matched normal tissue for comparative exomic analysis, a true over-abundance of mutations, or both is not clear. This is a known limitation of the sequencing approach in this setting, and previous exome sequencing studies performed in cell lines have shown similar results. Many of these mutations may have accumulated as cells continuously

### Table 3

<table>
<thead>
<tr>
<th>NCI-H295R</th>
<th>SW-13</th>
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<tbody>
<tr>
<td>Pathways in cancer (7)</td>
<td>Pathways in cancer (8)</td>
</tr>
<tr>
<td>PI3K-Akt signaling pathway (6)</td>
<td>Viral carcinogenesis (8)</td>
</tr>
<tr>
<td>HTLV-I infection (6)</td>
<td>MicroRNAs in cancer (7)</td>
</tr>
<tr>
<td>MicroRNAs in cancer (5)</td>
<td>HTLV-I infection (7)</td>
</tr>
<tr>
<td>Viral carcinogenesis (5)</td>
<td>PI3K-Akt signaling pathway (6)</td>
</tr>
</tbody>
</table>

Pathways shown in italics; number of mutated pathway members shown in parentheses.
evolve to acclimatize to conditions in culture, and thus likely may not represent or participate in the disease process of interest. Future functional validation of these variants will determine if they have significant implications for exploratory or therapeutic investigations or are merely an artifact of growth in culture. Our results should be applicable to investigators using these cell lines at relatively early passage, corresponding to the time of sequencing in this study; investigators utilizing long time points with many passages for their experiments will still need to be vigilant for the development of additional mutations in culture.

To summarize, this study confirms that the NCI-H29SR and SW-13 cell lines represent separate but overlapping molecular pathways in adrenocortical carcinogenesis and that these distinct mutational profiles reflect the genomic landscapes of previously established subsets of ACCs in patient-based studies. In particular, the genetic profiles of these cell lines share features in common with aggressive subsets of ACC tumors, potentially raising their value in the development of therapeutic agents for those refractory cases that they resemble in the present analysis. That said, even with the similarities reported here, results from cell culture and even animal models must always be interpreted with caution. Experiments in these systems will hopefully provide valuable direction for development of novel approaches, but the gold standard remains placebo-controlled in vivo human studies. The data presented here will nonetheless enable future investigators to use ACC cell lines more efficiently and effectively – with a clearer understanding of their genetic backgrounds – as model systems for this rare but aggressive tumor.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/JME-18-0262.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
N N conceived of the study, carried out data analysis and wrote the manuscript. R K conceived of the study, carried out data analysis and revised the manuscript. T C conceived of the study, supervised data analysis and revised the manuscript.

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